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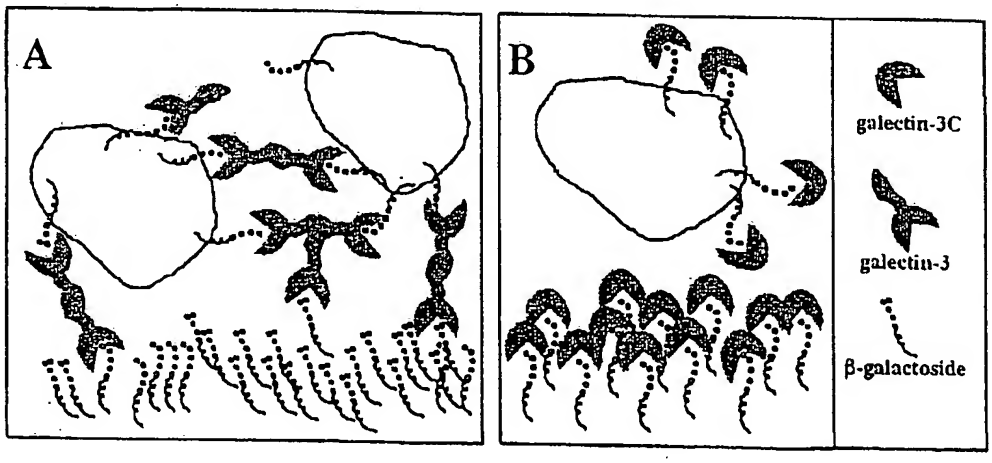
(54) Title: SUSTAINED RELEASE *N*-TERMINALLY TRUNCATED GALECTIN-3 AND ANTIBIDIES TO GALECTIN-3 CARBOHYDRATE LIGANDS FOR USE IN TREATING CANCER



(57) Abstract: There is provided a composition having an effective amount of *N*-terminally truncated galectin-3 in a pharmaceutically acceptable carrier or an effective amount of a nucleic acid sequence encoding *N*-terminally truncated galectin-3 in a pharmaceutically acceptable carrier. Also provided by the present invention is a method of treating cancer by administering to a patient in need of such treatment an effective amount of *N*-terminally truncated galectin-3 in a pharmaceutically acceptable carrier or an effective amount of a nucleic acid sequence encoding *N*-terminally truncated galectin-3 in a pharmaceutically acceptable carrier. The present invention also provides an antibody that specifically binds to carbohydrate ligands of galectin-3. Further, there is provided an anti-cancer treatment having an effective amount of *N*-terminally truncated galectin-3 and a pharmaceutically acceptable carrier or an effective amount of nucleic acid sequence encoding *N*-terminally truncated galectin-3 in a pharmaceutically acceptable carrier.

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SUSTAINED RELEASE N-TERMINALLY TRUNCATED GALECTIN-3 AND ANTIBODIES TO GALECTIN-3 CARBOHYDRATE LIGANDS FOR USE IN TREATING CANCER

BACKGROUND OF THE INVENTION

1. TECHNICAL FIELD

The present invention relates to methods and compositions for treating cancer. More specifically, the present invention relates to a composition containing galectin-3 for treating cancer.

2. BACKGROUND ART

There is evidence that tumor cell metastasis is, in part, due to complex intercellular interactions involving adhesion and aggregation. Specific classes of tumor proteins, lectins, which play a role in cell adhesion, are now known to be important in tumor formation and metastasis.

Lectins are, by definition, proteins with at least one carbohydrate-binding domain. By immobilizing monosaccharides, oligosaccharides, or glycoproteins in affinity columns, lectins have been isolated from tumor tissue extracts. Generally, a tissue extract in acetone or the like is utilized to isolate the protein component from the lipid component. The acetone is then evaporated, whereupon the residue is solublized in a buffered aqueous solution. This solution is then passed through an affinity column containing the immobilized carbohydrates or glycoproteins. A number of lectins, which selectively bind to galactosides, have been isolated in this manner.

Galectin-3 is one member of the family of lectins termed galectins, formerly known as S-type or S-Lac lectins. Galectins are classified as such due to their structural similarity and characteristic affinity for β-galactoside sugars (1, 2). The highest levels of galectin-3 are found in activated macrophages, basophils, mast cells, some epithelial cells, and sensory neurons. An early observation was that many tumor cells express galectins

on their surface and that their expression could be involved in adhesion and invasion processes. Experimental evidence also suggested that these galectins could be cross-linked by an exogenous glycoprotein resulting in the aggregation of tumor cells. Based on these results, Raz and Lotan proposed that galectin-1 and galectin-3 could promote tumor metastasis (3). Since that time, the evidence for the role of galectin-3 in tumor adhesion, invasion and metastasis has mounted.

Galectin-3 is composed of three distinct structural motifs: a short amino terminal region of 12 amino acids, a sequence rich in Gly-X-Tyr tandem repeats characteristic of the collagen supergene family, and a carboxy-terminal half containing the globular carbohydrate recognition domain (CRD) (2, 4, 5). There is close homology between the galectin-3 proteins of different species, but the number of *N*-terminal tandem repeats differs, hence, the sizes of the proteins vary (6). The human protein is composed of 250 amino acid residues with a M_r of ~31,000 based on electrophoretic mobility (7), and a CRD that extends from residue 117 to 250 (8). The X-ray crystal structure of the galectin-3C protein, beginning with amino acid residue Gly-108, complexed with lactose and *N*-acetyllactosamine has been published. This revealed that the CRD involves hydrogen binding of specific amino acid residues, the first being Arg-144 and the last being Glu-184 (8). The first residue with electron density was Leu-114, hence, the first 6 residues were presumed to be disordered (8).

A study of the minimal folding domain of galectin-3, required for lactose binding, was accomplished using a peptide library formed by cloning galectin-3 cDNA into a phage vector. The library was screened by affinity selection using lactose immobilized on agarose beads. DNA sequence analysis, of the clones isolated by their affinity for immobilized lactose, defined the minimal domain as about 136 amino acid residues, beginning with Ile-115 and extending through Ile-250 (9). Further deletions of 10 or 15 amino acids from either end abolished lactose binding and stable expression in bacteria. It is possible that lactose binding can be retained with deletions of fewer than 10

amino acid residues especially since the Ile-115 residue was not involved in direct interaction with lactose in the crystal structure. However, there was electron density indicative of such interaction around the carboxyl-terminal Ile-250 (8).

Although all galectins bind lactose with similar affinity, each galectin is more specific and has higher affinity for certain more complex saccharides (10, 11). Galectins, in general, are unusual among extracellular proteins in that they are initially mainly cytosolic but can be secreted by non-classical pathways, translocated to the cell nucleus, and endocytosed and trancytosed by cells. Galectins are thought to interact with various cell-surface and extracellular glycoproteins and glycolipids, thereby playing a role in cell adhesion, migration, and signaling. The relationship between the intra- and extracellular functions of galectins can be of great biological importance. A number of reviews of the biology of the galectins have been published (12-16).

Studies of mutants of hamster galectin-3 with various deletions in the N-terminal domain have shown that the even if lacking the first 103 amino acid residues the protein is localized in the nucleus. Deletion of the first 110 amino acid residues, however, prevented nuclear localization, although the exact sequence of amino acid residues 104-110, APTGALT, was not obligatory and substitution of other unrelated sequences permitted nuclear sequestration (17). The amino acid residues 104-110 of the hamster galectin-3 protein, according to the consensus sequence, correspond to the amino acid residues 109-115 of the homologous human galectin-3 protein (17). Thus, galectin-3C possibly could be localized in the nucleus as it is missing only the first 107 amino acids, if it is reuptaken by cells as is galectin-3. Loss of nuclear localization of galectin-3 was observed in senescent human fibroblasts (18), and binding to synexin was found to be associated with its nuclear transport (19).

Galectin-3 shares the ability to be secreted despite the absence of a signal peptide with a number of other proteins that have unconventional

intercellular transfer. These proteins are internalized by cells and are able to directly access the cytoplasm and the nucleus by a process that does not involve classical endocytosis (20). This is in contrast with the modulation of intercellular events by second messengers that bind to extracellular receptors and initiate a cascade of intracellular events that often involve transcriptional regulation. Although the mechanisms for the ability of some proteins to cross biological membranes in the absence of a signal sequence are poorly understood, a number of common features have been identified. Many of the proteins can directly access the nucleus, their mechanisms for secretion often vary from their mechanisms for entry, and apolipoproteins and cholesterol can play a role (20).

Galectin-3 is isolated as a monomer but undergoes multimerization upon binding to surfaces that contain glycoconjugate ligands. The *N*-terminal half of the protein is required for this property (21, 22). The *N*-terminal domain of the protein is required for galectin-3 to have affinity for multivalent carbohydrate ligands property (21, 23) and to transmit intracellular signals (24, 25). Galectin-3 promotes binding of cells to laminin and fibronectin, but the *N*-terminally truncated protein does not promote such binding (26). Thus, the *N*-terminal domain appears to be necessary for the self-association of galectin-3 that is required for some of its biological functions. Galectin-3 null cells were transfected to express recombinant galectin-3 and induced tumors within four weeks when injected into mice. When the same galectin-3 null cells were transfected to express a mutant galectin-3 that was lacking the 11 amino terminal amino acids, no tumors developed within four weeks (24).

A number of laboratories have studied the biology of galectin-3 that apparently is significant in cell growth, differentiation, adhesion, RNA processing, apoptosis, and malignant transformation (27). Laminin is the major non-collagenous polypeptide of basement membranes, and galectin-3 binds preferentially to mouse tumor laminin compared to human placental laminin (28). Galectin-3 has been shown to increase the binding of breast cancer cells to other extracellular matrix proteins (29, 30). In addition to

increasing the binding of tumor cells to basement membranes, the interaction of cell surface galectin-3 with complementary serum glycoproteins appears to promote aggregation of tumor cells in circulation, thereby playing another important role in the pathogenesis of metastasis (31).

Expression of recombinant galectin-3 in weakly metastatic fibrosarcoma cells resulted in an increased incidence of experimental lung metastases in syngeneic and nude mice (32). In human umbilical vein endothelial cells (HUVEC) galectin-3 induces angiogenesis (33). Increased expression of galectin-3 in human colon cancer cells resulted in increased metastases, and reduction in galectin-3 expression from antisense DNA was associated with decreased liver colonization and spontaneous metastasis in athymic nude mice (34). Exogenous galectin-3 has been shown to increase invasiveness of human breast cancer cells (35), and to be a chemotactic factor for human umbilical vein endothelial cells (34). Introduction of human galectin-3 cDNA into the human breast cancer cells BT-549, which are galectin-3 null and non-tumorigenic in nude mice, resulted in the establishment of four galectin-3 expressing clones, three of which acquired tumorigenicity when injected into nude mice (36). Nonetheless, the role of galectin-3 in cancer is complicated, and a number of different laboratories have found that decreased expression of galectin-3 is associated with increased tumorigenicity and metastasis (37-40). Overall, the body of work regarding the biochemistry and function of galectin-3 provides a strong rationale for continued exploration of its therapeutic use in cancer.

Galectin-3 is not a member of the Bcl-2 family of proteins, but at residues 180-183 it contains the four amino acid motif (NWGR) conserved in the BH1 domain of the Bcl-2 family, and it has 48% sequence similarity with Bcl-2 (41). Galectin-3 has anti-apoptotic activity that is abrogated by substitution of the Gly182 residue with Ala in the NWGR motif (42, 43). In T cells galectin-3 interacts with Bcl-2 in a lactose inhibitable manner and confers resistance to apoptosis induced by anti-Fas antibody and staurosporine (41). Galectin-3 has been found to improve cellular adhesion

and prevent apoptosis induced by loss of cell anchorage (anoikis) (43-45). Contact with the extracellular matrix is required for suppression of apoptosis of epithelial cells from a number of tissues.

By providing a mechanism for adherence of tumor cells to one another and to the extracellular matrix (26) and the subsequent suppression of apoptotis, galectin-3 on the surface of tumor cells appears to contribute to tumor invasion and metastasis. This premise is supported by the inhibition of spontaneous metastasis in a rat prostate cancer model by oral administration of modified citrus pectin, a complex polysaccharide rich in galactosyl residues. Citrus pectin (pH modified), a plant fiber component, can directly bind galectin-3, and can interfere with carbohydrate-mediated cell-cell and cell-matrix interactions (46).

U.S. Patent No. 5, 681,923, to Platt (1997), discloses the active site of galactose binding proteins. Two different peptide sequences are claimed that correspond to part of galectin-3 are claimed. One peptide is composed of 25 amino acids corresponding to residues 171 to 196 of galectin-3. The second peptide is 38 amino acids and corresponds to residues 158 to 196 of galectin-3. In both peptides there is a histidine instead of aspartic acid 178 of galectin-3. The X-ray crystal structure of the human galectin-3 carbohydrate recognition domain, in complex with lactose and *N*-acetyllactosamine, has been published (8). The carbohydrate recognition domain of galectin-3 extends from amino acid 117 to 250 with some of the residues between 144 and 184 directly involved in hydrogen binding to lactosaminylated substrates (8). It would instead be useful to develop a larger peptide region for use in treating cancer. A larger fragment would be likely to be more effective than the peptides that were disclosed in the Platt patent.

U.S. Patent No. 5,801,002, to Raz (1998), discloses sequences for the human galectin-3 protein. However, there is not disclosure regarding use of inhibition of the multimerization of galectin-3 that is required for many of the biological functions of the protein in cellular adhesion and signaling as a treatment. The *N*-terminal domain of the protein is critical for the

multimerization of galectin-3 when it is bound to carbohydrate ligands. Thus, it could be useful to develop a fragment of galectin-3 having a deleted *N*-terminal region so that it would still have the carbohydrate recognition domain and would still be able to bind to carbohydrate ligands for use in treating patients. This is because the *N*-terminally truncated galectin-3 does not have the ability to cross-link cells with other cells and to the extracellular matrix. The Raz patent describes using peptides as therapeutic agents that correspond to at least 4 consecutive amino acid residues of galectin-3. However, in the Raz patent there is no indication that one or another of these would be more or less effective in the inhibition of metastasis.

U.S. Patent 5,895,784, to Raz *et al.* (1999), discloses the use of pH-modified citrus pectin to treat cancer. Additionally, the Raz *et al.* patent describes the function, structure, and expression of galectin-3. The inventors indicated that they used citrus pectin to study the properties of galectin-3 and claimed a method of treating cancer by oral administration of modified pectin that can bind to the carbohydrate domain of galectin-3 to reduce metastasis. There is no disclosure of a truncated form of galectin-3 that prevents tumorigenicity and metastasis.

WO 98/122139 (PCT/US97/21807), to Huflejt et al. (1998), discloses the detection of human galectin-4 in various samples. Although the focus is on galectin-4, some of the methods described include the use of galectin-3 as controls. Again, there is no disclosure of a truncated form of galectin-3 that prevents tumorigenicity and metastasis.

U.S. Patent No. 5, 837,493, to Hillman *et al.* (1998), discloses some description of galectin-1, -2, and -3 including descriptions of the lectin structures and amino acid sequences. The sequence of the galectins and the use of galectins in the prevention of disease are disclosed. . However, there is no disclosure of a truncated form of galectin-3 that prevents tumorigenicity and metastasis.

The regulatory mechanism that produces the variable localization of galectin-3 in different cell types is not understood, and the significance of the

relative amounts of the protein found in the cytoplasm, or nucleus, or extracellular matrix of various cell types in terms of functionality is not understood. Many laboratories have studied the role of galectin-3 in cancer, and although the results of the studies are somewhat confusing they do indicate that galectin-3 is significant in some types of cancer. However, the prior art is lacking in a methodology and a composition of galectin-3 that can be successfully used to treat cancer. It would therefore be useful to develop molecules, methods, and compositions based on an *N*-terminally truncated form of galectin-3 that inhibit multimerization and carbohydrate binding of galectin-3 and can be used successfully to reduce tumor growth and metastasis.

SUMMARY OF THE INVENTION

According to the present invention, there is provided a composition having an effective amount of *N*-terminally truncated galectin-3 in a pharmaceutically acceptable carrier or an effective amount of a nucleic acid sequence encoding *N*-terminally truncated galectin-3 in a pharmaceutically acceptable carrier. Also provided by the present invention is a method of treating cancer by administering to a patient in need of such treatment an effective amount of *N*-terminally truncated galectin-3 in a pharmaceutically acceptable carrier or an effective amount of a nucleic acid sequence encoding *N*-terminally truncated galectin-3 in a pharmaceutically acceptable carrier. The present invention also provides an antibody that specifically binds to carbohydrate ligands of galectin-3. Further, there is provided an anti-cancer treatment having an effective amount of *N*-terminally truncated galectin-3 and a pharmaceutically acceptable carrier or an effective amount of a nucleic acid sequence encoding *N*-terminally truncated galectin-3 in a pharmaceutically acceptable carrier.

DESCRIPTION OF THE DRAWINGS

Other advantages of the present invention are readily appreciated, as the same becomes better understood by reference to the following detailed description when considered in connection with the accompanying drawings wherein:

Figure 1 is a schematic showing a postulated mechanism of action of *N*-terminally truncated galectin-3 whereby the truncated form of the protein inhibits the binding of intact galectin-3 to carbohydrate ligands and thereby also inhibits the multimerization and cross-linking activities of galectin-3;

Figure 2 is a graph showing the number of cells that have bound to laminin in the presence of galectin-3, *N*-terminally truncated galectin-3 (galectin-3C), or a mixture of both galectin-3 and the *N*-terminally truncated galectin-3; the number of cells was determined by a standard curve for the absorbance at 450 nm due to metabolism of WST-1 by a known number of MDA-MB-435 cells;

Figure 3 is a graph showing the toxicity of the therapy by plotting the weight change of the animals over time; Figure 3*A* shows body weights of animals determined weekly during the course of the therapy study and data is presented as the mean and standard deviation of the body weights of the treatment and control groups; Figure 3*B* shows body weight gains of animals exclusive of tumor weights at termination of therapy study, the body weights of the animals and their tumor weights were determined at the conclusion of the study and the weight of the tumor was subtracted from the final weight of the animal to calculate body weight gain; the black portion of the bar represents the mean body weights of mice in the treatment and control groups exclusive of tumor weights, whereas the gray portion of the bar represents the mean weights of tumors from mice in the treatment and control groups, the difference in body weight between the two groups of mice exclusive of tumor weight was not statistically significant ($P = 0.303$);

Figure 4 presents pharmacokinetic analysis of the intramuscular administration of galectin-3C determined at 2-12 hours; mice were injected

with ^{35}S-labeled galectin-3C and at the indicated time points the animals were sacrificed and serum and levels of radioactivity were determined; data is presented as the mean and standard deviation of the radioactive counts detected in five mice at each time point; the inset shows the pharmacokinetic analysis of the intravenous and intramuscular administration of galectin-3C was determined at eight hours; *p=0.033 when compared with cpm associated with blood cells (*t*-test);

Figure 5 shows the organ biodistribution analysis of the subcutaneous and intravenous administration of galectin-3C; mice were injected with ^{35}S-labeled galectin-3C and at eight hours post injection the animals were sacrificed and the heart, lungs, liver, kidneys, and spleen were removed and the amount of associated radioactivity was determined; background radioactivity was determined by measuring radioactive counts associated with organs from mice injected with vehicle only; background counts for each organ were subtracted from the presented data; data is given as the mean and standard deviation of the percentage of the dose injected that was detected in the organs of five mice;

Figure 6 is a graph of tumor volume over time showing the effect of galectin-3C treatment on the growth of primary orthotopic xenograph tumors in nude mice; mice were injected with either galectin-3C or vehicle control and the primary tumors were measured with calipers once per week after initiation of the treatment through the end of the 90 day treatment period; Tumor volume was calculated by the formula $W^2 \times L/2$, where W is the smallest dimension;

Figure 7 is a bar graph showing the effect of galectin-3C treatment on the weight of primary orthotopic xenograph tumors in nude mice; mice were injected with either galectin-3C or vehicle control and the weight of the primary tumors was measured at the end of the 90 day treatment period;

Figure 8 is graph of a repeated measures statistical model of the effect of galectin-3C treatment on the growth of primary orthotopic xenograph tumors in nude mice; a SAS Proc Mixed analysis was used to fit a linear

mixed model with random effects for cage and mouse slope and intercept; the fitted model was quadratic on treatment day; plot of the fitted model for log tumor volume versus treatment day shows steady growth for the control group but regressive growth for the galectin-3C group;

Figure 9 is a graph showing the mean tumor volume versus the days of treatment with either the control, *N*-terminally truncated galectin-3 or galectin-3;

Figure 10 is a series of photographs showing the efficacy evaluation of *N*-terminally truncated galectin-3 against the GFP-Gene Transfected Human Breast Cancer MDA-MB435 in a mouse model;

Figures 11 A-D are photographs showing a representative histopathology of primary tumor and lymph node, liver and lung metastasis in mice treated with the control;

Figures 12 A-D are photographs showing a representative histopathology of primary tumor and lymph node, lung, and liver metastasis in mice treated with the control;

Figures 13 A-C are photographs showing a representative histopathology of primary tumor and lymph node, and lung metastasis in mice treated with *N*-terminally truncated galectin-3;

Figures 14 A-C are photographs showing a representative histopathology of primary tumor, lymph node, and lung metastasis in mice treated with *N*-terminally truncated galectin-3; and

Figures 15 A-C are photographs showing a representative histopathology of primary tumor, lymph node, and lung metastasis in mice treated with galectin-3;

Figures 16 A-E are diagrams of polyethylene glycol diol with two free hydroxyls, monomethoxypolyethylene glycol or mPEG with a single reactive hydroxyl, mPEG-succinimidyl propionate that reacts with amino groups such as in lysine residues to produce stable amide linkages, and the sulfhydryl-selective PEGs, mPEG-maleimide and mPEG2-maleimide; and

Figure 17 is a diagram representing the reaction between the sulfhydyl on a cysteine residue of a protein and mPEG-maleimide.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a method and composition for treating cancer by administering an effective amount of *N*-terminally truncated galectin-3. Preferably the *N*-terminally truncated galectin-3 is formulated for sustained or slow-release. The *N*-terminally truncated galectin-3 is placed in a pharmaceutically acceptable carrier prior to administration. The present invention also provides a method and composition for treating cancer by administering an effective amount of antibody that specifically binds to carbohydrate ligands of galectin-3.

The composition of the present invention includes a sequence encoding the *N*-terminally truncated galectin-3 and analogues and homologues thereof and a pharmaceutically acceptable carrier. The composition can be used as a gene therapy.

Generally, the present invention provides a chemical entity being *N*-terminally truncated galectin-3 that binds lactose and that has been derivatized with one or more molecules of polyethylene glycol (PEG). The present invention provides a chemical entity being *N*-terminally truncated galectin-3 to which has been added one or more cysteine residues that have been also derivatized with PEG. Additionally, a class of chemical entities are provided these being antibodies that bind to complex saccharide ligands of galectin-3 and competitively inhibits the carbohydrate binding and, thereby, multimerization of galectin-3. Also provided is a class of chemical entities including *N*-terminally truncated galectin-3 that has been formulated for sustained release using methodologies that are well known to those skilled in the art.

In accordance with the present invention, there is provided an *N*-terminally truncated galectin-3 lacking the *N*-terminal 107 amino acids. The

N-terminally truncated galectin-3 was produced by enzymatic cleavage of the recombinant protein followed by collection of the pharmacologically active fragment, in the preferred method, and that carries PEG. Alternative methods of producing the *N*-terminally truncated galectin-3 can also be used such methods are known to those of skill in the art.

Also, there are provided *N*-terminally truncated human galectin-3 molecules that differ slightly in length. These molecules can be somewhat longer or shorter than the 143 amino acid residue *N*-terminally truncated galectin-3 that is lacking the *N*-terminal 107 amino acids. The molecules have essentially the same ability to inhibit the carbohydrate binding and multimerization of galectin-3 and, therefore, inhibit tumorigenicity and metastasis *in vivo*. The *N*-terminally truncated human galectin-3 molecules that differ slightly in length also are derivatized with polyethylene glycol and are formulated for sustained or slow-release and placed in a pharmaceutically acceptable carrier.

The present invention provides *N*-terminally truncated galectin-3 molecules that contain fusion tags on the *C*- or *N*-terminus. Examples of the tags include, but are not limited to, the commercially available His-Tag ® (Novagen, Madison, WI) for pET vector constructs that can contain 6, 8, or 10 histidine residues. These vectors can be used with more than a dozen different fusion tags that possess a variety of unique binding qualities and endoprotease sites that allow for high yields and a variety of methods in that they can be used to increase the purity of recombinant protein. The *N*-terminal tags can be cleaved off using enterokinase from Novagen, Roche (Indianapolis, IN) or Sigma (St. Louis, MO) or other similar compounds as are known to those of skill in the art. A *C*-terminal histidine$_6$ tag can be used for immobilized metal-affinity chromatography. *C*-terminal histidine$_6$ tag is not immunogenic, does not alter the affinity of most proteins, and has been used in both preclinical animal and human clinical trials (47). The methods for producing these slightly different versions of the *N*-terminally truncated human galectin-3 protein are known to those of skill in the art. Other pET

vector expression systems available from Novagen, such as pET3, which contain restriction enzyme sites that are used to produce the desired product without any fusion tags, can also be used in accordance with the present invention.

The present invention provides the C-terminal domain of galectin-3 for use as a therapeutic agent. Preferably, the therapeutic agent is modified to be in a slow release form. Also provided are antibodies that bind to the complex saccharide ligands of galectin-3 and that act to inhibit the carbohydrate binding and multimerization of the protein as does the N-terminally truncated galectin-3.

Proteins possess unique chemical and physical properties and the manufacture, formulation, and delivery of proteins represent significant challenges to pharmaceutical scientists (48). Proteins traditionally are administered by inconvenient modes such as intravenous injection or infusion, or subcutaneous injection and must be given frequently due to rapid degradation. Recent advances in clinically useful, sustained-release formulations for proteins allow less frequent administration. Examples of sustained-release formulation include, but are not limited to, injectable microspheres that encapsulate protein (49, 50). One such product approved for human use, is manufactured by Nutropin Depot and is produced by encapsulating human growth factors in biodegradable microspheres. This formulation requires only one or two doses a month, but can require more than one injection per dose. Preparation of microspheres by lyophilization of a protein-PEG aqueous mixture has been reported for sustained release formulation (51). Polymers approved for use in humans for sustained release from biodegradable microsphere formulations include, but are not limited to, polylactides [poly(lactic acid), poly(glycolic acid), and poly(lactic-coglycolic) acid (52). Twice daily injections of the *N*-terminally truncated galectin-3 molecule were used in the mouse model of metastatic human breast described in Example 1. Formulation of the *N*-terminally truncated galectin-3 in a slow release formulation reduces the frequency of administration required

and therefore increases the convenience of the method, increases the efficacy of the treatment by increasing the levels that could be sustained in the body by the treatment, and reduces the cost. Methods for making such formulations are known to those of skill in the art.

More than two decades have passed since the first descriptions of the effect of the covalent attachment PEG on the circulating life and immunogenicity of proteins (53). A PEGylated version of interferon-alpha by Schering-Plough Corporation was approved by the F.D.A. last year for use in the U.S. for chronic hepatitis C. Administration of the PEGylated version of interferon-alpha is subcutaneous injection once weekly rather than three times weekly.

Many methods and PEG derivatives are available for conjugation of proteins to PEG (54-56). PEG modification of antibodies to tumors has been found to enhance penetration into the tumors and to increase the anti-tumor effects (57). The circulating lives of single chain antibodies have been extended by conjugation with PEG to the carboxylic acid groups or the primary amines. An increase in the polymer length rather than total mass was found to be more effective for serum half-life extension (58).

Specific PEG derivatives react with thiols such as the amino acid residue cysteine (56, 59). These include PEG-ortopyridyl-disulphide, PEG-malemide, and PEG-vinylsulphone. In a preferred method, *N*-terminally truncated human galectin-3 that is lacking the 107 amino acids on the N-terminus or is similar in size is derivatized on the single cysteine in the sequence (SEQ ID NO. 1). Alternatively, the N-terminally truncated human galectin-3 can be produced with one or more cysteine residues on the *N*-terminus as described above (SEQ ID NO. 2). Then one or more of the cysteine residues is derivatized with a thiol reactive PEG derivative. Employing a branched PEG derivative and performing the derivatization in the presence of ligand can prevent active site residue derivatization due to steric hindrance (56).

As described above, there is close homology between the galectin-3 proteins of different species, but the number of *N*-terminal tandem repeats differs, hence, the size of the proteins vary (6). The epitopes of murine monoclonal antibodies generated to human galectin-3 were all found to be within the first 45 amino acid residues of the N-terminus (18). Attempts to obtain polyclonal antibody that bound to the *N*-terminally truncated galectin-3 lacking the first 107 amino acids as described in Example 1 also were unsuccessful. It can be difficult to produce antibody to the carbohydrate recognition domain because of the homology between the galectins from different species.

The *N*-terminally truncated galectin-3 competitively inhibits the binding of galectin-3 to carbohydrates in the nucleus, cytoplasm, extracellular matrix and in cell-cell adhesions, and acts to prevent tumorigenicity and metastasis. The non-carbohydrate binding *N*-terminal domain of galectin-3 promotes multimerization of the protein, and enables it to cross link cancer cells to the matrix and other cells (21, 22). The *N*-terminal half of the protein is the most critical for homophilic interactions, although there is evidence that the carbohydrate recognition domain can contribute to cross-linking (60-62).

Excess administered *N*-terminally truncated galectin-3, in which the carbohydrate binding part of the protein has been removed, occupies the binding sites of endogenous galectin-3. The *N*-terminally truncated galectin-3 itself has little or no cross-linking activity and acts as a dominant-negative inhibitor of galectin-3. Therefore, *N*-terminally truncated galectin-3 prevents the homophilic cross-linking of galectin-3 and other types of protein-protein binding interactions that promote tumorigenicity and metastasis.

Antibodies that bind to the carbohydrate ligands of galectin-3 also have this same effect. Studies of the specificity of galectin-3 have been performed (10, 11, 63, 64). Galectin-3 has specific affinity for polyNAc-lactosaminoglycan, a polymer of β(1,3)-linked LacNAc units that occurs on cell surfaces and extracellular matrices. Oligosaccharides of the specific sequences recognized by galectin-3 can be conjugated and used to induce antibody production. The

ability to produce antibody to oligosaccharide antigens is well established. This technique is used in immunotherapy using tumor-associated antigens to induce production by the patient's body of antibodies to tumors (65, 66). Bispecific antibodies that are specific for a particular tumor type or tissue can be used to target the therapy (67-69). Generally, those of skill in the art know methodology for induction, isolation, and purification of both polyclonal and monoclonal antibodies.

The *N*-terminal domain is not required for oligosaccharide binding, but is necessary for positive cooperativity (21, 23). The result of the positive cooperativity is that multimers of galectin-3 bind to substrate surfaces (21). Thus, galectin-3 multimers can cross-link a tumor cell with the extracellular membrane. The C-terminal fragment competitively inhibits the binding of galectin-3 to substrates.

More specifically, soluble recombinant *N*-terminally truncated galectin-3 effectively competes with endogenous galectin-3 for carbohydrate binding sites in the extracellular matrix and cell-cell adhesions important in tumor invasion and metastasis. The *N*-terminal domain of galectin-3 promotes multimerization of the protein, and enables it to cross-link cancer cells to the matrix and other cells. Excess administered *N*-terminally truncated galectin-3, in which the *N*-terminal part of the protein has been removed, occupies binding sites of endogenous galectin-3 and prevents its cross-linking activities. *N*-terminally truncated galectin-3 itself does not have significant cross-linking activity since it lacks the *N*-terminal part of galectin-3, and acts like a dominant-negative inhibitor of galectin-3.

Recombinant *N*-terminally truncated galectin-3 is efficacious for inhibition of tumor invasion and metastasis in cancer. The mechanism shown in Figure 1 is the competitive inhibition by recombinant galectin-3 of the binding of the galectin-3 on the surface of metastatic cancer cells to laminin and other β-galactoside glycoconjugates in the extracellular matrix. The *N*-terminal domain of galectin-3 promotes its multimerization and, thus, enables it to cross-link cancer cells to the matrix and to other cells. Excess

administered truncated galectin-3, in which the *N*-terminal part of the galectin-3 has been removed, occupies the galectin-3 carbohydrate binding sites in the extracellular matrix and in cell-cell adhesions important in tumor invasion and metastasis. This truncated version of galectin-3 itself does not have cross-linking activity since it lacks the *N*-terminal domain of galectin-3. Hence, *N*-terminally truncated galectin-3 acts like a dominant-negative inhibitor of galectin-3 and prevents galectin-3 mediated binding of cells to the extracellular matrix and cell-cell adhesion as shown in Figure 1. This concept is supported by the fact that galectin-3 itself, but not the *N*-terminally truncated galectin-3 produced by collagenase digestion, promotes binding of cells to laminin and fibronectin (26). Thus, the *N*-terminally truncated galectin-3 molecule by blocking the multimerization of the intact protein prevents the adhesion of tumor cells with one another and with the extracellular matrix. In addition to preventing adhesion to the extracellular matrix and, thus, metastasis, prevention of the contact of cancer cells with the extracellular matrix can also lead to their programmed cell death or apoptosis that is induced by loss of cell anchorage (also called anoikis) (43, 44).

The amount of sustained release or PEGylated *N*-terminally truncated galectin-3 or antibody, which is utilized in the composition of the present invention, is a sufficient amount to at least reduce tumor size. Alternatively, it can prevent or reduce metastasis of a tumor. The tumors being treated can include breast cancer, prostate cancer, colon cancer, lung cancer, and all additional solid and liquid forms of cancer.

Alternatively, the compound of the present invention can be useful in preventing tumor growth. In such an example, by maintaining the titers of sustained release form or PEGylated *N*-terminally truncated galectin-3 or antibodies to galectin-3 saccharide in the body of an individual, the composition can prevent tumor growth by preventing tumors from forming in the first place. As stated in the Background Art, it is known that lectins could be useful in treating tumors and preventing metastasis, accordingly, by creating a preventative treatment that prevents the long term growth of tumor

cells in the body, the compound of the present invention can be useful in preventing initial tumor growth. The amount of composition for treatment is based upon the body weight of the individual being treated and can be determined by individuals of skill in the art.

The present invention provides a method of treating cancer in a patient by administering to a patient in need of such treatment an effective amount of *N*-terminally truncated galectin-3 that has been derivatized with one or more molecules of polyethylene glycol (PEG). A second method of treating cancer in a patient is provided that is by administering to a patient an effective amount of *N*-terminally truncated galectin-3 to which has been added one or more cysteine residues and that has been derivatized with PEG. A third method of treating cancer in a patient is provided that is by administering to a patient an effective amount of an antibody that binds to complex saccharide ligands of galectin-3. A fourth method of treating cancer in a patient is provided that is by administering to a patient an effective amount of *N*-terminally truncated galectin-3 that has been formulated for sustained release using methodologies that are well known to those skilled in the art.

The "effective amount" for purposes herein is thus determined by such considerations as are known in the art of cancer treatment wherein it must be effective to provide measurable improvement in persons given the treatment, and, in a preferred embodiment, complete recovery of the patient without the presence of cancer cells.

In order to effectuate the treatment of the present invention, there is administered to a patient an effective amount of the sustained release form of the *N*-terminally truncated galectin-3 or antibody to galectin-3 saccharide ligands in a pharmaceutically acceptable carrier. Administration can occur intramuscularly, orally, intravenously, locally, subcutaneously, or in any other applicable mechanism known to those of skill in the art. The mechanism of treatment varies depending upon the cancer that is being treated and can be best determined by those of skill in the art.

For example, *N*-terminally truncated galectin-3 was evaluated as a potential therapeutic agent for breast cancer based on the lectin galectin-3. It was determined that therapy with an *N*-terminally truncated form of galectin-3 is efficacious for inhibition of metastases. Recombinant galectin-3 was produced and the *N*-terminally truncated galectin-3 was derived by collagenase enzyme digestion and affinity chromatography. Injected *N*-terminally truncated galectin-3 was detected by metabolic labeling with ^{35}S methionine prior to collagenase cleavage. As shown in Figure 3 the maximum tolerated dose of *N*-terminally truncated galectin-3 in nude mice was determined to be greater than 125 mg/kg without overt adverse effects. The pharmacokinetic elimination half-life of *N*-terminally truncated galectin-3 administered intramuscularly into nude mice was found to be 3.01 hours in the serum and 4.26 in the cellular fraction of the blood. Mice bearing orthotopically implanted tumors derived from breast cancer cell line MDA-MB435 was treated intramuscularly twice daily for 90 days with *N*-terminally truncated galectin-3 or a vehicle control. It was found that the mean tumor volumes and weights were statistically significantly less in mice treated with *N*-terminally truncated galectin-3 compared with control mice, and that fewer numbers of mice exhibited lymph node metastases in the treated group compared with the control group. It was therefore concluded that *N*-terminally truncated galectin-3 is not overtly toxic and is efficacious in reducing metastases and tumor volumes and weights in primary tumors.

Additionally, the compounds of the present invention can be utilized in a combination therapy. This can include adding to the pharmaceutically acceptable carrier additional chemotherapeutic compounds for further treatment of the cancer cells.

Compositions for treating cancer are provided by the present invention. There is provided a composition having *N*-terminally truncated galectin-3, with or without one or more cysteines added to the *N*-terminus, and that has been derivatized with PEG and that is in a pharmaceutically acceptable carrier. A composition is provided consisting of an antibody that binds to the

complex saccharide ligands of galectin-3 and that is in a pharmaceutically acceptable carrier. Another composition provided by the present invention has *N*-terminally truncated galectin-3 that is derivatized with another chemical or formulated for sustained release and that is in a pharmaceutically acceptable carrier.

The compound of the present invention is administered and dosed in accordance with good medical practice, taking into account the clinical condition of the individual patient, the site and method of administration, scheduling of administration, patient age, sex; body weight and other factors known to medical practitioners. The pharmaceutically "effective amount" for purposes herein is thus determined by such considerations as are known in the art. The amount must be effective to achieve improvement including but not limited to improved survival rate or more rapid recovery, or improvement or elimination of symptoms and other indicators as are selected as appropriate measures by those skilled in the art.

It is noted that humans are treated generally longer than the mice or other experimental animals exemplified herein which treatment has a length proportional to the length of the disease process and drug effectiveness. The doses can be single doses or multiple doses over a period of several days, but single doses are preferred.

The doses can be single doses or multiple doses over a period of several days. The treatment generally has a length proportional to the length of the disease process and drug effectiveness and the patient species being treated.

One method for producing the *N*-terminally truncated human galectin-3 includes first producing intact recombinant human galectin-3 that can be produced in *Escherichia coli* Bl21 (DE3) transformed with pET3cGal3 (the pET3c plasmid containing the human galectin-3 coding DNA) as described previously (21). Next, the bacteria can be lysed by sonication and the galectin-3 protein can be purified by affinity chromatography on lactosyl-Sepharose (21) and dialyzed to remove lactose. The product is then cleaved

with *Clostridium histiolyticum* collagenase type VII (Sigma Chemical Co., St. Louis, MO). Specifically, the recombinant galectin-3 is incubated overnight at 37°C with a lectin:collagenase ratio of 20:1 (by weight) in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1 mM $CaCl_2$. The resulting *N*-terminally truncated galectin-3 can be purified by affinity chromatography on lactosyl-Sepharose. For storage and shipment, a procedure can be used involving dialysis against water followed by lyophilization. The dry *N*-terminally truncated galectin-3 powder can be stored at –20°C for various amounts of time, up to three months, the time being determined by those of skill in the art. The retention of the carbohydrate binding activity of an aliquot can be ascertained by testing on a small lactosyl-Sepharose column. This establishes that the *N*-terminally truncated galectin-3 can be stored and shipped as a lyophilized powder without losing activity.

The amino acid sequence of the *N*-terminally truncated recombinant human galectin-3 that is produced by exhaustive digestion with collagenase, and that can be produced by other cloning methods is designated as SEQ ID NO. 1, and is as follows:

gap agplivpynl
plpggvvprm litilgtvkp nanrialdfq rgndvafhfn prfnennrrv ivcntkldnn
wgreerqsvf pfesgkpfki qvlvepdhfk vavndahllq ynhrvkkine isklgisgdi
dltsasytmi

The amino acid sequence of the *N*-terminally truncated recombinant human galectin-3 produced by cloning methods to contain one additional cysteine on the *N*-terminus is designated as SEQ ID NO. 2, and is as follows:

cgap agplivpynl
plpggvvprm litilgtvkp nanrialdfq rgndvafhfn prfnennrrv ivcntkldnn
wgreerqsvf pfesgkpfki qvlvepdhfk vavndahllq ynhrvkkine isklgisgdi
dltsasytmi

The amino acid sequence of the intact recombinant human galectin-3 described by Oda *et al* (46) is designated as SEQ ID NO. 3, and its sequence is as follows:

1 madnfslhda lsgsgnpnpq gwpgawgnqp agaggypgas ypgaypgqap pgaypgqapp
61 gayhgapgay pgapapgvyp gppsgpgayp ssgqpsapga ypatgpygap agplivpynl
121 plpggvvprm litilgtvkp nanrialdfq rgndvafhfn prfnennrrv ivcntkldnn
181 wgreerqsvf pfesgkpfki qvlvepdhfk vavndahllq ynhrvkkine isklgisgdi
241 diisasytmi

A plasmid containing the complete galectin-3 coding sequence can be used as a template in a PCR reaction using primers designed to amplify the desired fragment.

Forward primer: 5' GACGACGACAAGGGCGCCCCTGCTGGG 3'

Reverse primer: 5' GAGGAGAAGCCCGGTTTATATCATGGTATA 3'

Underlined sequences in each of the primers match the plasmid sequences for pET32 (EK/LIC expression system, Novagen, Madison, WI). The reverse primer defines the *C*-terminal protein sequence and does not differ in these procedures. The non-underlined portion of the forward primer defines the *N*-truncated version of the native galectin-3 that in this example begins with Gly-108 ("delta 1-107", starting at amino acid sequence glycine, alanine, proline, alanine, etc.). The underlined sequences are added as tails and are used to fuse the PCR product with the pET32 Ek/LIC plasmid using the Ek/LIC ligation protocol (Novagen, Madison, WI). This particular plasmid produces a fusion protein with a variety of unique binding qualities and endoprotease sites allowing for high yields and purity of the recombinant protein. More than one cysteine can be introduced to the construct by simply including more cysteine codons (either *tgt* or *tgc*) to create a version of *N*-truncated galectin-3 having one or more cysteines where they should not interfere with cabohydrate binding, for example, at the *N*- or C-terminus.

E. coli BL21(DE3) bacteria are transformed with the above-described construct and the bacteria can be used for protein production. Expression is under the control of bacteriophage T7 transcription and can be induced by providing a source of T7 RNA polymerase, such as infection with a phage that carries the T7 RNA polymerase gene or moving the plasmid into a cell

containing an expression host containing a copy of the T7 RNA polymerase gene.

The above discussion provides a factual basis for the use of the compositions of the present invention. The method used with and the utility of the present invention can be shown by the following non-limiting examples and accompanying figures.

EXAMPLES:

General methods in molecular biology: Standard molecular biology techniques known in the art and not specifically described were generally followed as in Sambrook et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, New York (1989), and in Ausubel et al., *Current Protocols in Molecular Biology*, John Wiley and Sons, Baltimore, Maryland (1989) and in Perbal, *A Practical Guide to Molecular Cloning*, John Wiley & Sons, New York (1988), and in Watson et al., *Recombinant DNA*, Scientific American Books, New York and in Birren et al (eds) *Genome Analysis: A Laboratory Manual Series, Vols. 1-4* Cold Spring Harbor Laboratory Press, New York (1998) and methodology as set forth in United States patents 4,666,828; 4,683,202; 4,801,531; 5,192,659 and 5,272,057 and incorporated herein by reference. Polymerase chain reaction (PCR) was carried out generally as in *PCR Protocols: A Guide To Methods And Applications*, Academic Press, San Diego, CA (1990). In-situ (In-cell) PCR in combination with Flow Cytometry can be used for detection of cells containing specific DNA and mRNA sequences ((70)

General methods in immunology: Standard methods in immunology known in the art and not specifically described are generally followed as in Stites et al.(eds), Basic and Clinical Immunology (8th Edition), Appleton & Lange, Norwalk, CT (1994) and Mishell and Shiigi (eds), Selected Methods in Cellular Immunology, W.H. Freeman and Co., New York (1980).

Immunoassays

In general, ELISAs are the preferred immunoassays employed to assess a specimen. ELISA assays are well known to those skilled in the art. Both polyclonal and monoclonal antibodies can be used in the assays. Where appropriate other immunoassays, such as radioimmunoassays (RIA) can be used as are known to those in the art. Available immunoassays are extensively described in the patent and scientific literature. See, for example, United States patents 3,791,932; 3,839,153; 3,850,752; 3,850,578; 3,853,987; 3,867,517; 3,879,262; 3,901,654; 3,935,074; 3,984,533; 3,996,345; 4,034,074; 4,098,876; 4,879,219; 5,011,771 and 5,281,521 as well as Sambrook et al, *Molecular Cloning: A Laboratory Manual*, Cold Springs Harbor, New York, 1989.

Antibody Production

Antibody Production: Antibodies can be monoclonal, polyclonal or recombinant. Conveniently, the antibodies can be prepared against the immunogen or portion thereof for example a synthetic peptide based on the sequence, or prepared recombinantly by cloning techniques or the natural gene product and/or portions thereof can be isolated and used as the immunogen. Immunogens can be used to produce antibodies by standard antibody production technology well known to those skilled in the art as described generally in Harlow and Lane, *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1988 and Borrebaeck, *Antibody Engineering - A Practical Guide*, W.H. Freeman and Co., 1992. Antibody fragments can also be prepared from the antibodies and include Fab, $F(ab')_2$, and Fv by methods known to those skilled in the art.

For producing polyclonal antibodies a host, such as a rabbit or goat, is immunized with the immunogen or immunogen fragment, generally with an adjuvant and, if necessary, coupled to a carrier; antibodies to the immunogen are collected from the sera. Further, the polyclonal antibody can be absorbed such that it is monospecific. That is, the sera can be absorbed against related immunogens so that no cross-reactive antibodies remain in the sera rendering it monospecific.

For producing monoclonal antibodies the technique involves hyperimmunization of an appropriate donor with the immunogen, generally a mouse, and isolation of spleenic antibody producing cells. These cells are fused to a cell having immortality, such as a myeloma cell, to provide a fused cell hybrid that has immortality and secretes the required antibody. The cells are then cultured, in bulk, and the monoclonal antibodies harvested from the culture media for use.

For producing recombinant antibody (see (71); (72); Mernaugh and Mernaugh, 1995), messenger RNAs from antibody producing B-lymphocytes of animals, or hybridoma are reverse-transcribed to obtain complimentary DNAs (cDNAs). Antibody cDNA, which can be full or partial length, is amplified and cloned into a phage or a plasmid. The cDNA can be a partial length of heavy and light chain cDNA, separated or connected by a linker. The antibody, or antibody fragment, is expressed using a suitable expression system to obtain recombinant antibody. Antibody cDNA can also be obtained by screening pertinent expression libraries.

The antibody can be bound to a solid support substrate or conjugated with a detectable moiety or be both bound and conjugated as is well known in the art. (For a general discussion of conjugation of fluorescent or enzymatic moieties see Johnstone & Thorpe, *Immunochemistry in Practice*, Blackwell Scientific Publications, Oxford, 1982.) The binding of antibodies to a solid support substrate is also well known in the art. (see for a general discussion Harlow & Lane *Antibodies: A Laboratory Manual*, Cold Spring Harbor Laboratory Publications, New York, 1988 and Borrebaeck, *Antibody Engineering - A Practical Guide*, W.H. Freeman and Co., 1992) The detectable moieties contemplated with the present invention can include, but are not limited to, fluorescent, metallic, enzymatic and radioactive markers such as biotin, gold, ferritin, alkaline phosphatase, b-galactosidase, peroxidase, urease, fluorescein, rhodamine, tritium, ^{14}C and iodination.

Delivery of gene products/therapeutics (compound):

The compound of the present invention is administered and dosed in accordance with good medical practice, taking into account the clinical condition of the individual patient, the site and method of administration, scheduling of administration, patient age, sex, body weight and other factors known to medical practitioners. The pharmaceutically "effective amount" for purposes herein is thus determined by such considerations as are known in the art. The amount must be effective to achieve improvement including but not limited to improved survival rate or more rapid recovery, or improvement or elimination of symptoms and other indicators as are selected as appropriate measures by those skilled in the art.

In the method of the present invention, the compound of the present invention can be administered in various ways. It should be noted that it can be administered as the compound or as pharmaceutically acceptable salt and can be administered alone or as an active ingredient in combination with pharmaceutically acceptable carriers, diluents, adjuvants and vehicles. The compounds can be administered orally, subcutaneously or parenterally including intravenous, intraarterial, intramuscular, intraperitoneally, and intranasal administration as well as intrathecal and infusion techniques. Implants of the compounds are also useful. The patient being treated is a warm-blooded animal and, in particular, mammals including man. The pharmaceutically acceptable carriers, diluents, adjuvants and vehicles as well as implant carriers generally refer to inert, non-toxic solid or liquid fillers, diluents or encapsulating material not reacting with the active ingredients of the invention.

It is noted that humans are treated generally longer than the mice or other experimental animals exemplified herein that treatment has a length proportional to the length of the disease process and drug effectiveness. The doses can be single doses or multiple doses over a period of several days, but single doses are preferred.

The doses can be single doses or multiple doses over a period of several days. The treatment generally has a length proportional to the length

of the disease process and drug effectiveness and the patient species being treated.

When administering the compound of the present invention parenterally, it is generally formulated in a unit dosage injectable form (solution, suspension, emulsion). The pharmaceutical formulations suitable for injection include sterile aqueous solutions or dispersions and sterile powders for reconstitution into sterile injectable solutions or dispersions. The carrier can be a solvent or dispersing medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, liquid polyethylene glycol, and the like), suitable mixtures thereof, and vegetable oils.

Proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and by the use of surfactants. Nonaqueous vehicles such a cottonseed oil, sesame oil, olive oil, soybean oil, corn oil, sunflower oil, or peanut oil and esters, such as isopropyl myristate, can also be used as solvent systems for compound compositions. Additionally, various additives that enhance the stability, sterility, and isotonicity of the compositions, including anti-microbial preservatives, anti-oxidants, chelating agents, and buffers, can be added. Prevention of the action of microorganisms can be ensured by various anti-bacterial and anti-fungal agents, for example, parabens, chlorobutanol, phenol, sorbic acid, and the like. In many cases, it is desirable to include isotonic agents, for example, sugars, sodium chloride, and the like. Prolonged absorption of the injectable pharmaceutical form can be brought about by the use of agents delaying absorption, for example, aluminum monostearate and gelatin. According to the present invention, however, any vehicle, diluent, or additive used is compatible with the compounds.

Sterile injectable solutions can be prepared by incorporating the compounds utilized in practicing the present invention in the required amount of the appropriate solvent with several of the other ingredients, as desired.

A pharmacological formulation of the present invention can be administered to the patient in an injectable formulation containing any compatible carrier, such as various vehicle, adjuvants, additives, and diluents; or the compounds utilized in the present invention can be administered parenterally to the patient in the form of slow-release subcutaneous implants or targeted delivery systems such as monoclonal antibodies, vectored delivery, iontophoretic, polymer matrices, liposomes, and microspheres. Examples of delivery systems useful in the present invention include: 5,225,182; 5,169,383; 5,167,616; 4,959,217; 4,925,678; 4,487,603; 4,486,194; 4,447,233; 4,447,224; 4,439,196; and 4,475,196. Many other such implants, delivery systems, and modules are well known to those skilled in the art.

A pharmacological formulation of the compound utilized in the present invention can be administered orally to the patient. Conventional methods such as administering the compounds in tablets, suspensions, solutions, emulsions, capsules, powders, syrups and the like are usable. Known techniques that deliver it orally or intravenously and retain the biological activity are preferred.

In one embodiment, the compound of the present invention can be administered initially by intravenous injection to bring blood levels to a suitable level. The patient's levels are then maintained by an oral dosage form, although other forms of administration, dependent upon the patient's condition and as indicated above, can be used. The quantity to be administered varies for the patient being treated and varies from about 100 ng/kg of body weight to 100 mg/kg of body weight per day and preferably is from 10 mg/kg to 10 mg/kg per day.

Example 1

As background for the following example, mounting evidence suggests that tumor cells express the β-galactoside-binding lectin galectin-3 on their surfaces and that tumor cells metastasize partly due to processes involving

cellular adhesion and aggregation mediated by galectin-3. Galectin-3 binds via its C-terminus carbohydrate recognition domain to binding sites in the extracellular matrix (1). The goal of this research was the evaluation of a potential therapeutic agent for breast cancer based on galectin-3 lectin that acts directly to reduce metastases. Soluble recombinant *N*-terminally truncated galectin-3 competes with endogenous galectin-3 for carbohydrate binding sites in the extracellular matrix and cell-cell adhesions important in tumor invasion and metastasis. The *N*-terminal domain of galectin-3 promotes multimerization of the protein, and enables it to cross link cancer cells to the matrix and other cells. Excess administered *N*-terminally truncated galectin-3, in which the *N*-terminal part of the protein has been removed, occupies binding sites of endogenous galectin-3 and prevents its cross-linking activities. *N*-terminally truncated galectin-3 itself has less cross-linking activity since it lacks the *N*-terminal part of galectin-3, and acts like a dominant-negative inhibitor of galectin-3, as shown in Figure 1. The experiments establish that therapy with recombinant *N*-terminally truncated galectin-3 is efficacious for inhibition of tumor invasion and metastasis in breast cancer. The overall purpose was to determine the efficacy, safety, and mechanism of action of *N*-terminally truncated galectin-3 in treatment of metastatic breast cancer using a nude mouse model of metastasis.

Preparation for animal studies.

The carbohydrate recognition domain of galectin-3 (*N*-terminally truncated galectin-3) was produced as described previously (21). High yield expression (20-300 mg/liter culture) of active soluble galectin-3 can be obtained. The intact recombinant galectin-3 was produced in *Escherichia coli* Bl21/DE3 containing the pET3c plasmid (Novagen) with the human galectin-3 coding DNA (pET3cGal3). Galectin encoding DNA can be amplified by PCR using galectin cDNA as template and primers that contain restriction sites for cloning of the product into the expression vector in the proper reading frame. The PCR product is first cloned into the TA-vector (Invitrogen, Carlsbad, CA),

the insert is released with the restriction enzymes corresponding to the primers, and it is finally cloned into the expression vector. The expression construct is used to transform the proper host strain (e.g. *E. coli* BL21 for the pET system). The organisms are lysed by sonication and the galectin-3 protein is purified by affinity chromatography on lactosyl-Sepharose (21). The purified galectin-3 is dialyzed to remove lactose and cleaved with *Clostridium histiolyticum* collagenase type VII (Sigma). The resulting *N*-terminally truncated galectin-3 is purified again by affinity chromatography on lactosyl-Sepharose.

For storage and shipment a new procedure was developed involving dialysis against water followed by lyophilization. The dry *N*-terminally truncated galectin-3 powder was stored at –20 °C for various amounts of time up to three months and the retention of the carbohydrate binding activity of an aliquot was ascertained by testing on a small lactosyl-Sepharose column. Other batches (with or without enrichment in ^{15}N) were analyzed by NMR-spectroscopy. This analysis confirmed that the protein had retained its proper folding. Therefore, *N*-terminally truncated galectin-3 can be stored and shipped as a lyophilized powder without loosing activity.

To produce ^{35}S labeled *N*-terminally truncated galectin-3 for pharmacokinetic studies, the plasmid pET3cGal3 was transfected into *E. coli* B834 (Novagen), which is a methionine auxiotroph derived from BL21/DE3. The *E. coli* was adapted for growth on M9 minimal medium supplemented with ampicillin (50 mg/ml) and methionine (40 mg/ml)(M9-Met) by passage on M9-Met plates three times. To produce ^{35}S galectin-3, a colony from the last plate was inoculated into 0.5 liters of M9-Met supplemented with 1.0 mCi ^{35}S-Met. The bacteria were cultured, induced with IPTG and harvested as described previously (21). To lyse the radioactive *E. coli*, sonication was avoided because of aerosol formation. Various alternative methods were tested and the following method was determined to be most efficient. To the bacterial pellet there was added 5 ml sucrose (25%) in 50 mM TrisHCl, pH 8.0 with 50 mM NaCl, 20 mM EDTA, and 8 mg lysozyme. After ten minutes on

ice, 16 ml water was added and the sample kept on ice another 30 minutes. The sample was centrifuged at 12000 rpm for 30 minutes and the supernatant applied to lactosyl-Sepharose. The galectin-3 was eluted, dialyzed and treated with collagenase to generate *N*-terminally truncated galectin-3 as described above.

Cellular Adhesion Assay

A cellular adhesion assay was performed that is similar to that described previously (26) for use in 96-well microtiter plates. The wells were coated with 50 microliters of 20 micrograms/ml of human laminin in PBS overnight at 4°C. The wells then were washed once with minimum essential media (MEM), and then blocked for 1 hour at 37°C with 1% R.I.A. grade BSA in MEM, washed once with MEM containing 0.1% Tween-20 and twice with MEM. To each well was added 4.5×10^4 human breast cancer cells, MDA-MB435 with various concentrations of galectin-3, the *N*-terminally truncated galectin-3, or a mixture of both proteins. Either 0, 5, 10, 15, or 20 micrograms per ml of galectin-3 or the *N*-terminally truncated protein were added in MEM. In a third set of wells, 20 micrograms per ml of *N*-terminally truncated galectin-3 was added with 5, 10, 15, or 20 micrograms per ml of galectin-3 in MEM. After incubation for 15 minutes at 37°C the nonadherent cells were removed and the wells gently washed with MEM. To each well was added 100 microliters of MEM and 10 microliters of WST-1 cell viability reagent (Roche, Mannheim, Germany) and the plate was placed in a CO_2 incubator at 37°C for 90 minutes. The UV absorbance of the samples was recorded at 450 nm subtracting the absorbance at 650 nm in a Molecular Devices Thermomax plate reader.

The results of the assay are shown in Figure 2. The increased number of cells adherent to laminin with increasing concentrations of galectin-3 is clearly revealed. Similarly the lack of significant effect of increasing concentrations of *N*-terminally truncated galectin-3 is also revealed and is similar to the previously reported data (26). Adding the *N*-terminally truncated

galectin-3 blocked the increase in cellular adhesion to laminin mediated by galectin-3. The data show the directly competitive effect of the *N*-terminally truncated protein on the cellular adhesion that is promoted by intact galectin-3, and supports the mechanism proposed in Figure 1.

Immunization of four chickens with purified, *N*-terminally truncated galectin-3 and purification of polyclonal Ig from chicken eggs.

Two chickens were immunized with purified *N*-terminally truncated galectin-3 and the polyclonal Ig was purified. The Ig should bind to both galectin-3 and *N*-terminally truncated galectin-3. When tested, the chicken polyclonal anti-N-terminally truncated galectin-3 was of very low affinity as determined by repeated nitrocellulose dot blots of galectin-3 and *N*-terminally truncated galectin-3 following lactose elution of each protein from a lactosyl-Sepharose column. The presence of galectin-3 and *N*-terminally truncated galectin-3 protein in specific fractions from the column was confirmed by the measurement of UV absorbance at 280 nm. Detection of anti-galectin-3 antibody (rat IgG) binding to galectin-3 was used as a positive control on a separate dot blot using anti-rat IgG labeled with alkaline phosphatase (AP). For the chicken polyclonal anti-N-terminally truncated galectin-3 antibody, an anti-chicken Ig antibody (Zymed, South San Francisco) labeled with biotin was used followed by AP-conjugated streptavidin and AP substrate. The results of these studies provided no evidence that immunization of chickens produces a high affinity antibody specific for *N*-terminally truncated galectin-3. Therefore, an alternative strategy of generating ^{35}S-labeled *N*-terminally truncated galectin-3 was used.

Pharmacokinetic analysis & determination of Maximum Tolerated Dose.

A dose determination study was carried out in non-tumor bearing female athymic nude mice in order to establish the MTD of *N*-terminally truncated galectin-3 using a single bolus dose. The dose finding study comprised four dose groups with each group consisting of five mice. The

subcutaneous doses administered were 1 mg/kg, 5 mg/kg, 25 mg/kg, and 125 mg/kg. In addition, a vehicle treated control group consisting of five mice was evaluated. No overt abnormal signs were observed within 48 hours of injection. Animals were observed for a total of five days after injection at which time body weight and viability were determined. The mean body weights for each group were statistically identical ($p>0.10$; t-test) at five days indicating that all doses of *N*-terminally truncated galectin-3 did not effect the normal physiological growth of the mice. From these results it was concluded that *N*-terminally truncated galectin-3 can be injected into nude mice at a dose as high as 125 mg/kg without overt adverse effects.

Pharmacokinetic analysis.

Analyses of the pharmacokinetic and biodistribution characteristics of *N*-terminally truncated galectin-3 were determined for intravenous and intramuscular routes of administration of *N*-terminally truncated galectin-3 into nude mice as shown in Figure 4. Groups of five mice (approximately 0.03 kg/mouse) were each injected with 150 μg/mouse (1 mg per ml; 5 mg/kg = dose) of a mixture of ^{35}S-labeled *N*-terminally truncated galectin-3 and unlabeled *N*-terminally truncated galectin-3 in a weight ratio of 1:9 (labeled:unlabeled). For the intramuscular route, the animals were sacrificed and blood samples were obtained by terminal cardiac puncture at four time points: 2 hours, 4 hours, 8 hours, and 12 hours after injection. In addition, blood samples were obtained from one control group of five animals 1 hour after injection of vehicle only (1 mg/ml lactose in PBS). Serum samples from 200 μl of blood were analyzed for radioactivity in triplicate. For a direct comparison of the pharmacokinetic characteristics of intravenous versus intramuscular administration of *N*-terminally truncated galectin-3, two groups of five mice each were injected with the mixture of ^{35}S-labeled *N*-terminally truncated galectin-3 and unlabeled *N*-terminally truncated galectin-3 either intravenously or intramuscularly. At eight hours post-injection serum and blood cell samples from 200 μl of blood were analyzed for radioactivity in

triplicate. In addition, the organ biodistribution of *N*-terminally truncated galectin-3 was determined in mice injected subcutaneously and intravenously with the mixture of ^{35}S-labeled *N*-terminally truncated galectin-3 and unlabeled *N*-terminally truncated galectin-3. At eight hours post-injection, the heart, lungs, liver, kidneys, and spleen were removed and the amount of associated radioactivity was measured.

During *the distribution phase* after an intravenous dose, changes in the concentration of drug are primarily due to movement of drug within the body. The distribution phase primarily determines the early rapid decline in plasma concentration of a drug after an intravenous dose. With time, equilibrium is reached in the distribution of the drug between the plasma and the tissues, and changes in plasma reflect proportional changes in all the other tissues. During the *elimination phase*, after the rapid decline of the distribution phase, the decline in plasma concentration is due only to elimination of the drug from the body and is characterized by the *elimination half-life* ($T_{1/2}$) (73, 74). The elimination half-life is the time it takes for the concentration of the drug in the plasma (and body) to be reduced by one-half. The apparent volume of distribution is the apparent volume of distribution of the drug in the body at equilibrium. The volume of distribution is equal to the amount of drug in the body at T_0 divided by the plasma drug concentration at T_0.

In a first-order elimination process the half-life is independent of the concentration of the drug in the body and the following equations apply.

Equation 1. $$T_{1/2} = \frac{0.693}{k}$$ (where k is the elimination rate constant)

Equation 2. $$k = \frac{2.303}{\text{Time(2)-Time(1)}} \times \log \frac{\text{conc}_{\text{Time(1)}}}{\text{conc}_{\text{Time(2)}}}$$

Calculation of the volume of distribution requires that distribution equilibrium be achieved between the drug in the tissues and the plasma. After administration, the amount of the drug in the body is equal to the dose but the distribution equilibrium has not yet been achieved. To estimate the

plasma concentration that would have resulted if the drug was immediately distributed into its final volume of distribution, the linear decline during the elimination phase, as shown in the semilogarithmic plot, is used. The pharmacokinetic analysis of the intramuscular administration of *N*-terminally truncated galectin-3 is shown in Figure 4. The serum elimination half-life (Figure 4A) and the elimination half-life of the cellular fraction of the blood (Figure 4B) were calculated by regression analysis of the linear portion of the curve between 2 and 12 hours as described above. Thus, for the intramuscular administration of *N*-terminally truncated galectin-3, the serum $T_{1/2}$ = **3.01 h** and the cellular fraction $T_{1/2}$ = **4.26 h.**

The eight hours of distribution of *N*-terminally truncated galectin-3 into serum versus blood cell compartments for *N*-terminally truncated galectin-3 administered intravenously and intramuscularly was compared. As shown in Figure 4C, both routes of administration resulted in the distribution of *N*-terminally truncated galectin-3 into serum and blood cells. For intravenous administration, the quantity of *N*-terminally truncated galectin-3 associated with blood cells was greater than that in serum ($p=0.03$; t-test). For intramuscular administration, there was not a statistically significant difference in the amount of *N*-terminally truncated galectin-3 localized to either compartment. These findings indicate that a portion of the administered *N*-terminally truncated galectin-3 is associated with blood cells and can serve as a reservoir for slow release in serum.

The organ biodistribution of *N*-terminally truncated galectin-3 was also investigated. Two groups of five mice were injected either subcutaneously or intravenously with the mixture of ^{35}S-labeled *N*-terminally truncated galectin-3 and unlabeled *N*-terminally truncated galectin-3. A third group of five mice was injected intravenously with the lactose in PBS vehicle. At eight hours post-injection, the heart, lungs, liver, kidneys, and spleen were removed and the amount of associated radioactivity was measured. As shown in Figure 5, the liver, kidney, and spleen were sources of *N*-terminally truncated galectin-3-associated radioactivity above the background vehicle level, whereas

neither the heart nor lungs localized any *N*-terminally truncated galectin-3. These data suggest that in addition to serum and blood cells, certain organs can localize *N*-terminally truncated galectin-3 and possibly serve as reservoirs for eventual systemic release. Alternatively, these organs can function in the ultimate removal of *N*-terminally truncated galectin-3 from circulation.

Comparison in the MetaMouse^R model of metastatic breast cancer of the efficacy of treatment with *N*-terminally truncated galectin-3 to control animals (vehicle only).

Female athymic CD-1 nude mice between four and five weeks of age were used in the study. The animals were bred and maintained in a HEPA-filtered environment with cages, food, and bedding sterilized by autoclaving. The breeding pairs were obtained from the Charles River Laboratories (Wilmington, MA). The animal diets were obtained from Harlan Teklad (Madison, WI). Ampicillin (Sigma) at a concentration of 5% (v/v) was added to the autoclaved drinking water.

Breast cancer cell line MDA-MB435, that expresses galectin-3 (75), was transfected with a plasmid expressing green fluorescent protein as previously described (76), and cells were injected into the subcutis of nude mice to form solid tumors. Test animals for the study were transplanted by surgical orthotopic implantation using fragments harvested from the subcutaneously growing tumors. The animals were anesthetized with isoflurane and the surgical area was sterilized using iodine solution and alcohol. An incision approximately 0.5 cm long was made on the second right mammary gland. The gland was then pulled out and two fragments of 1 mm^3 of MDA-MB435-GFP tumor tissue were sutured onto the gland with a sterile nylon 8-0 surgical suture. The skin incision was closed with a sterile silk 6-0 surgical suture. All surgical and animal manipulations and procedures were conducted under HEPA-filtered laminar flow hoods.

Extra numbers of mice were transplanted to compensate for possible postsurgical losses and tumor non-takes. The orthotopically-transplanted animals used for the study were selected to establish groups of similar mean tumor size and body weight. Groups for each of the cohort conditions were randomly chosen. The cohort study was grouped as shown in Table 1 for a total of 45 mice. In addition, the production of 50 mg of galectin-3 allowed the testing of five mice with the intact protein. Administration of the treatments was begun when tumors reached palpable sizes. Based on the calculated elimination half-life of *N*-terminally truncated galectin-3 administered intramuscularly, the dosing schedule for the vehicle control and *N*-terminally truncated galectin-3 groups was twice a day for the intramuscular injections with an approximately 6-8 hour interval between injections for a total 90 days. The dosing for galectin-3 was once a day intramuscular injections. Both *N*-terminally truncated galectin-3 and galectin-3 were injected as solutions of concentration 1 mg/ml in PBS containing 1 mg/ml lactose. The vehicle control was PBS containing 1 mg/ml lactose.

Table 1. Efficacy Test Study Design

Group	Dose	Dosing Schedule	Number of Mice
Vehicle Control	125 µl/dose	im, bid x 90 days	20
N-terminally truncated galectin-3	125 µg/dose	im, bid x 90 days	20
Galectin-3	110 µg/dose	im, qd x 90 days	5

Data Collection and Results:

Body weight and animal survival - Animal weight was determined by an electronic balance once a week during the course of the efficacy test. Figure 3 shows the mean body weights of the mice over time in each of the three treatment groups. The mean body weight of the mice in the control group at day 90 was 30.4 ± 2.89 g, whereas that of the *N*-terminally truncated galectin-3 treatment group was 28.6 ± 1.91 g. This represents a statistically significant

difference in the body weights between the two groups (p=0.026; t-test). The likely explanation for this finding is that the control group had tumors that weighed more than the tumors in the *N*-terminally truncated galectin-3 group as described below (tumor weight: control mean 2.34 g vs. treated mean 1.25 g). The tumor-free mean body weight changes of the test and control groups were statistically identical (p=0.303). In addition, the difference in body weights between the control group and the galectin-3 treated group was not statistically significant.

All animals in all three groups survived the 90 day course of treatment. Thus, no overt toxicity was observed for treatment of mice with either *N*-terminally truncated galectin-3 or galectin-3 for a period of 90 days.

Primary tumor volume and weight - The primary tumors were measured by a pair of calipers once a week from initiation of treatment through the end of the study. The mean tumor volume over time for galectin-3C compared to the vehicle-only control group is shown in Figure 6, and the galectin-3 treated group is included in Figure 9. Table 2 shows a comparison of the mean tumor volumes of the mice in each group at the end of the treatment regime. The mean tumor volume measured in the mice treated with *N*-terminally truncated galectin-3 was significantly less than that in the control mice (p=0.003), whereas the mean tumor volume in mice treated with galectin-3 was similar to that in the control mice (p=0.865). Using a whole-body optical imaging system, the growth of the GFP-expressing tumors was also visualized in real time. Figure 10 shows imaging analyses of the 90-day efficacy test of *N*-terminally truncated galectin-3. Tumor fragments of breast cancer cell line MDA-MB-435 expressing green fluorescent protein were orthotopically implanted into the breast pad of nude mice. Real time, quantitative measurement of tumor growth, metastases, and micrometastases were performed using whole-body optical imaging. The images are representative of the external images of the development of the breast tumor.

Upon autopsy, all visible primary tumors were removed and weighed using an electronic balance. Table 3 shows a comparison of the mean tumor weights at autopsy of mice in each treatment group. The mean tumor weights of the vehicle-only control and the galectin-3C treated groups are shown graphically in Figure 7. The mean tumor weight in the mice treated with *N*-terminally truncated galectin-3 was significantly less than that in the control mice (p=0.007), whereas the mean tumor volume in mice treated with galectin-3 was similar to that in the control mice (p=0.634).

Table 2. Mean tumor volumes at autopsy*

Group	Number of Mice	Mean primary tumor volume (mm^3) ± SD	P value**
Vehicle Control	20	2368.4 ± 1732.7	-
N-terminally truncated galectin-3	20	1149.2 ± 1679.7	0.003
Galectin-3	5	2307.1 ± 1718.0	0.865

*Tumor volume was calculated by the formula W^2 x $L/2$, where W is the smallest dimension.
**All treated groups compared to vehicle control by the Mann-Whitney U test.

Table 3. Mean tumor weights at autopsy

Group	Number of Mice	Mean primary tumor weight (g) ± SD	P value*
Vehicle Control	20	2.34 ± 1.47	-
N-terminally truncated galectin-3	20	1.25 ± 1.65	0.007
Galectin-3	5	2.30 ± 1.44	0.634

*All treated groups compared to vehicle control by the Mann-Whitney U test.

Assessment of metastasis: At autopsy, tissue samples from the auxiliary lymph node, the liver, and the lungs were collected and processed through standard procedures of hematoxylin and eosin staining for subsequent microscopic examination. Representative histopathology of the primary

tumors in each group is shown in Figures 11-15. No significant pathological differences within primary tumors were noted among the groups.

Metastasis to the auxiliary lymph node, the lung, and the liver was assessed microscopically. Representative histopathology of the metastatic tumors in each group is shown in Figures 11-15. Table 4 shows the results of this assessment. Statistical analyses were carried out using the Chi-square/Fisher exact test. Eleven out of twenty mice had auxiliary lymph node metastasis in the control group whereas only four of the twenty mice developed auxiliary lymph node metastasis in the *N*-terminally truncated galectin-3 group by the end of the study ($p<0.05$). The incidence of metastases in the liver and the lung between the *N*-terminally truncated galectin-3 group and the control group was not different. Treatment with galectin-3 did not demonstrate a significant difference in metastatic incidence from the control. Tables 5 and 6 show the metastatic data as a function of tumor volumes categorized as small, medium, and large. A comparison of the two Tables reveals that five out of the eight small tumors in the control group had associated metastases while 0 of the 15 small tumors in the *N*-terminally truncated galectin-3 group had associated metastases. This shows that a reduction in tumor volume by *N*-terminally truncated galectin-3 results in a decreased incidence of metastases.

Table 4. Incidence of metastases in lymph node, lung and liver

Group	Number of Mice	Lymph Node	P value*	Lung	P value	Liver	P value
Vehicle Control	20	11/20	-	3/20	-	2/20	-
Truncated galectin-3	20	4/20	0.022	4/20	1.0	0/20	0.487
Galectin-3	5	1/5	0.322	1/5	1.0	0/5	1.0

*All treated groups compared to vehicle control by the Chi-square/Fisher exact test.

Table 5. Number of metastases in control group as a function of tumor volume

Metastatic assessment	Small tumors (0-1500 mm^3)	Medium tumors (1501-2999 mm^3)	Large tumors (>2999 mm^3)
metastases	5 (LN only)	2 (LN only)	4*
no metastases	3	5	1

*All 4 mice had lymph node metastases; 2 of those had liver and lung metastases, and 1 of those had lung metastases

Table 6. Number of metastases in *N*-terminally truncated galectin-3 group as a function of tumor volume

Metastatic assessment	Small tumors (0-1500 mm^3)	Medium tumors (1501-2999 mm^3)	Large tumors (>2999 mm^3)
metastases	0	2 (2 lung; 0 liver)*	2 (2 lung; 0 liver)*
no metastases	15	1	0

*Both mice had lymph node and lung but not liver metastases

In treated animals the number of primary tumors that metastasized was less than controls by a factor of 2.75 ($p = 0.022$), and the mean primary tumor weights of the treated were approximately 2-fold less by the end of the 90-day study ($p = 0.007$).

Statistical methods used in efficacy evaluation (77): Animal body weight comparisons were performed by Student's t-test. The primary tumor volume and weight were evaluated by the Mann-Whitney U test. The incidence of metastasis in the lymph node, lung, and the liver were evaluated by the Chi-square/Fisher-exact test. All tests were two-sided with $\alpha = 0.05$.

In addition to the statistical data analyses of the efficacy data described above, an analysis of tumor volume growth over time was performed using a repeated measures statistical model for log tumor volume, with cage in random statement. Specifically, this was a SAS Proc Mixed analysis to fit a linear mixed model with random effects for cage and mouse slope and intercept. The fitted model was quadratic in treatment day. The model was applied to the vehicle control and *N*-terminally truncated galectin-3 treatment data for tumor volume that showed a significant difference at day 90 when tested with the Mann-Whitney U test. The model allowed us to sensitize the analysis by factoring out non-treatment parameters such as cage and mouse

variation, tumor volume trajectory differences within a group, and residual unexplained variation.

As shown in Figure 8, the mean tumor volume in the group of mice treated with *N*-terminally truncated galectin-3 is statistically significantly less than that in the vehicle control group during more than 50% of the treatment period. At day 45 of treatment, which is labeled as TxDayCenter 0 on the x-axis, the slope of the tumor volume curve for the *N*-terminally truncated galectin-3 group is significantly different than that of the vehicle control group ($p=0.045$; SAS type 3 F test). Following day 45, treatment with *N*-terminally truncated galectin-3 resulted in increased differences between the slopes and trajectories of the two lines representing the mean tumor volumes of the two groups of mice. This model strongly supports the conclusion that *N*-terminally truncated galectin-3 was efficacious in reducing the number of metastases and as well as tumor growth over time in the orthotopic implantation mouse model of breast cancer.

CONCLUSIONS

The maximum tolerated dose of *N*-terminally truncated galectin-3 in nude mice at five days was determined to be greater than 125 mg/kg without overt adverse effects. This suggested that the safety threshold for the use of *N*-terminally truncated galectin-3 *in vivo* is high. The pharmacokinetic analysis of the intramuscular administration of ^{35}S-labeled *N*-terminally truncated galectin-3 into nude mice indicated an elimination half-life of *N*-terminally truncated galectin-3 of 3.01 hours in serum and 4.26 hours in the cellular fraction of the blood. The effort to produce antibody to the carbohydrate recognition domain of human galectin-3 in chickens was not successful very likely due to the homology in this domain of galectin-3 among various species. Organ biodistribution analyses showed that *N*-terminally truncated galectin-3 localized to the liver, kidney, and spleen but not to the heart or lungs.

Based on the pharmacokinetic and maximum tolerated dose data and the amount of *N*-terminally truncated galectin-3 that was produced, mice were treated twice daily with *N*-terminally truncated galectin-3 for 90 days and the results were compared with the treatment efficacy of mice treated with vehicle only. At the end of the treatment regime, it was found that the mean tumor volume and mean tumor weight were statistically significantly less in the group of mice treated with *N*-terminally truncated galectin-3 compared with the group of mice treated with the vehicle control. Repeated measures of the statistical model for tumor growth over time showed that the mean tumor volume was statistically significantly less by day 45 of the 90 day treatment regime in the group of mice treated with *N*-terminally truncated galectin-3 compared with the group of mice treated with the vehicle control. In addition, there were statistically significantly fewer numbers of mice that had lymph node metastases in the group of mice treated with *N*-terminally truncated galectin-3 compared with the vehicle control group of mice.

The hypothesis that was tested was that therapy with recombinant *N*-terminally truncated galectin-3 is efficacious for inhibition of tumor invasion and metastasis in breast cancer. Taken together, the data generated in this study strongly support this hypothesis. It was found that *N*-terminally truncated galectin-3 was efficacious in reducing breast cancer metastases in the orthotopic implantation mouse model of breast cancer. The average volume of the tumors in the treated group was less than that of the vehicle-only control group beginning on day 14 until the end of the study, reaching statistical significance by day 63 using the T test to compare the groups. The mean tumor weight of the galectin-3C treated group upon necropsy was less than the control group ($p = 0.007$) by a factor of almost two-fold. Only two animals in the treated group had large tumors of a size greater than 2,999 mm^3, whereas 5 animals in the control group had tumors of this size. These data support the conclusion that treatment of breast tumor-bearing mice with galectin-3C significantly reduced the progression of tumor growth.

The result of the SAS Proc Mixed analysis also strongly supports the conclusion that galectin-3C was efficacious in reducing the number of metastases as well as actually shrinking tumors and inhibiting tumor growth over time. Further evidence is gained by the fact that in the treated group in some individual mice tumor growth slowed and tumor volume regressed, and that there was no primary tumor observed in 3 treated animals (15%) upon necropsy at the end of the 90-day study.

In general, orthotopically transplanted tumors are more clinically relevant than subcutaneous xenograft models due to the physiological location of the tumor and the spontaneous hematogenous metastases (78). Treatment with galectin-3C reduced the percentage of animals with metastases from 55% (11/20) in the control group to 20% (4/20) in the treated group, a decrease of 2.75-fold. None of the animals in the treated group with small tumors less than 1500 mm^3 in size (0/15) had metastases, whereas 62.5% (5/8) of the control group that had tumors this size had metastases. Only one animal in the treated group had a tumor larger than 1500 mm^3 that had not metastasized compared to 6 animals in the control group that had tumors of this size that had no metastases. Apparently animals in the treated group that had metastatic tumors were not responding to galectin-3C as their tumors were larger as well.

Importantly, it was found that *N*-terminally truncated galectin-3 also reduced tumor volume and tumor weight in the primary tumors. The mechanism of action of extracellular *N*-terminally truncated galectin-3 was likely to be at least partly due to: 1) decreased homotypic aggregation and adhesion of breast cancer cells to extracellular matrices and endothelial cells; 2) decreased chemotaxis (galectin-3 itself is a chemotactic factor) (79); or 3) anoikis (apoptosis induced by loss of cell anchorage) (43).

The development of a system for detection of *N*-terminally truncated galectin-3 in nude mice using ^{35}S-labeled *N*-terminally truncated galectin-3 allowed the accurate determination of the *in vivo* pharmacokinetics of intramuscularly injected *N*-terminally truncated galectin-3. The fact that *N*-

terminally truncated galectin-3 exhibited no detectable toxicity and reduced tumor volume, tumor weight, and incidence of metastases in a nude mouse model of breast cancer strongly supports the use of *N*-terminally truncated galectin-3 as a therapeutic compound for the treatment of breast cancer and other types of cancer. The results indicate that galectin-3C has minimal overt toxicity and that it has utility in treatment of breast cancer by shrinking tumors and reducing tumor growth over time, and by inhibiting metastasis.

Alternatively, an antibody to the specific complex carbohydrate ligands of galectin-3 has a longer half-life than galectin-3C and inhibits galectin-3 binding and multimerization as does the *N*-terminally truncated galectin-3 protein. Such an antibody also increases the efficacy obtainable. Thus, the rationale for either a sustained release form of the protein or an antibody to galectin-3 carbohydrate ligands is the same, to increase the efficacy, cost-effectiveness, and convenience of the therapy.

Breast cancer is the most frequently diagnosed cancer in women in the U.S., other than non-melanoma skin cancer that has a much lower mortality rate, according to figures from the American Cancer Society. Despite the available treatments breast cancer ranks second for women as the cause of death from cancer, and the lifetime risk of breast cancer for women living in the U.S. is now 1 in 8. Among those factors predictive of risk of recurrence of breast cancer are age, tumor size, hormone receptor status, and histologic type and grade, metastatic carcinoma in axillary lymph nodes is more important than any other currently available (80, 81). Metastatic breast cancer has a poor prognosis especially if the tumor is hormone-independent, and is the primary cause of mortality in individuals already affected. Fewer than 10% of patients with metastatic breast cancer survive 5 years or more (82). There is evidence that galectin-3 plays a role in other types of cancer including thyroid, colon, and prostate, hence, these types of cancer are also susceptible to treatment with galectin-3C.

Example 2

As background for the following example, the first PEGylated protein that was approved for use in the United States by the Food and Drug Administration was PEG-adenosine deaminase (PEG-ADA), for patients with severe combined immunodeficiency disease. Second to be approved was PEGylated asparaginase, used for the treatment of acute lymphoblastic leukemia. Also, the PEGylated version of interferon-alpha has been approved for human use (83).

Most proteins are cleared from the circulation by the reticuloendothelial system (RES), kidney, spleen, or liver. Clearance depends on the size, charge, glycosylation, and cellular receptors of the protein. Metabolism by proteases and peptidases also can lead to loss of biological activity and degradation. Modification of proteins with PEG can extend their half-life from 3 to 486-fold (84). The extension of half-life can be partly due to increasing the molecular weight of the modified protein until it is large to make the cut-off for glomerular filtration, that is somewhere between molecular mass 67 and 68 kDa (84), thus, necessitating addition of a 40 or 50 kDa PEG to a small protein. Larger proteins are often cleared by the RES or specific cellular receptors, and this clearance also can be reduced by PEGylation. Modification with PEG also can reduce proteolytic degradation.

A drawback of PEGylation can be reduced biological activity and this has been found to occur with many proteins (56, 85). Derivatization of the active site of an enzyme or a residue in a biologically important binding domain of a protein can lead to inactivation. Site-specific modification a one or a few residues of the protein can be used to avoid this problem. Steric hindrance obtained with a bulky PEG derivative can be used to decrease reactivity at some sites. Performing the derivatization reaction in the presence of ligand or substrate can decrease reactivity of some amino acid residues. Lowered stoichiometry of PEG derivatives can also be exploited to alter product ratios. The pH of the reaction can be altered in derivatization with amino reactive PEGs to limit their reactivity.

One method for forming derivative proteins is to use PEGs that react with nitrogens on lysine or terminal amines to form stable amide linkages, such as mPEG-succinimidyl propionate or butanoate. With reagents of this type, the use of a basic pH in the reaction can result in a mixture of products with PEGylation at all lysine residues where lower pH can limit the derivatization to a single residue. Site-specific modification of a single His residue of interferon-alpha-2B was achieved using a reaction with succinimidyl carbonyl PEG at pH 5.4 (86). Using N-hydroxysuccinimidyl carboxymethyl at pH 5, PEG enabled site-specific modification of the N-terminus of human granulocyte stimulating factor (87).

One method for derivatizing proteins with PEG is to use thiol-reactive PEGs that forms covalent bonds with cysteine residues, such as mPEG-maleimide (Shearwater Corporation, Huntsville, AL) (56, 57, 59). The endogenous cysteine (Cys-173 of human galectin-3) of *N*-terminally truncated galectin-3 can be used as a site for covalent modification with PEG. Alternatively, using genetic engineering, one or more additional cysteines can be added to either the *C*- or the *N*-terminal end of the *N*-terminally truncated galectin-3 protein. The cysteines can be used as sites to react with the thiol-reactive PEGS. The *N*-terminally truncated galectin-3, with and without additional cysteines added by genetic engineering, can be produced by cloning methods.

The relative short half-life of the intramuscularly injected *N*-terminally truncated galectin-3, the lack of acute toxicity of higher doses, and the efficacy demonstrated by the 4 mg/kg dose given twice daily provides clear rationale for formulation of a sustained release form of the protein, for example using a PEG derivatization, to increase the half-life. The sustained release form enables higher levels of the protein to be sustained in the body and thereby obtains greater efficacy with more convenience and less expense. One method to achieve site specific derivatization is to use sulfhydryl reactive PEG derivatives as shown in Figure 15 and 16. The *N*-

terminally truncated galectin-3, with and without additional cysteines added by genetic engineering, can be produced by known cloning methods.

A plasmid containing the complete galectin-3 coding sequence is used as a template in a PCR reaction using primers designed to amplify the desired fragment.

Forward primer: 5' GACGACGACAAG*tgc*GGCGCCCCTGCTGGG 3'

Reverse primer: 5' GAGGAGAAGCCCGGTTTATATCATGGTATA 3'

The reverse primer defines the carboxy-terminal protein sequence and in this example does not differ from that required to produce recombinant human galectin-3. The non-underlined portion of the forward primer 5'-GACGACGACAAG*tgc*ggcgcccctgctgggccactg-3' introduces a cysteine immediately upstream of the *N*-truncated sequence providing an additional site on which PEG can be attached (starting first with a cysteine then continuing with the rest of the amino acid sequence glycine, alanine, proline, alanine, etc.). The underlined sequences are added as tails that can be used to fuse the PCR product with pET32 Ek/LIC plasmid using the Ek/LIC ligation protocol (Novagen, Madison, WI). This plasmid produces a fusion protein with a variety of unique binding qualities and endoprotease sites allowing for high yields and purity of the recombinant protein. More than one cysteine can be introduced to the construct by simply including more cysteine codons (either *tgt* or *tgc*) to create a version of *N*-truncated galectin-3 with multiple cysteines. One or more cysteines can be added to either the *C*- or *N*-terminus for derivatization with PEG using this method. The site of PEGylation can effect the carbohydrate binding or the biological localization of the truncated galectin-3 molecules and the bioactivity of the molecule can vary accordingly. The relative affinity for carbohydrate ligands and the localization of derivatized truncated galectin-3 molecules can be determined readily by those with skill in the art using standard methodologies.

The E. coli BL21 (DE3) bacteria is transformed with this construct and can be used for protein production. Expression is under the control of bacteriophage T7 transcription and is induced by providing a source of T7

RNA polymerase, such as infection with a phage that carries the T7 RNA polymerase gene or moving the plasmid into a cell containing an expression host containing a copy of the T7 RNA polymerase gene.

Cloning.

The PCR products are purified using spin columns, and then ligated into pET32 following the vendor's instructions. The ligated product is transformed into competent Novablue bacteria and clones are selected based on blue-white selection. DNA was extracted from picked clones and analyzed by gel electrophoresis, PCR, and sequencing. The plasmid is sequenced using the same forward and reverse primers as PCR using an ABI 3700 machine. This DNA is used to transform BL21 strain of *E. coli.* Isolated colonies were picked, grown in culture, and glycerol stocks are stored for future use.

Growing Bacteria.

Luria-Bertani (LB) broth with ampicillin (50 mg/l) was inoculated with 20 µl/L of glycerol frozen stock of transformed *E. coli.* The culture is shaken at 37°C overnight. Then, the inducer, isopropyl-β-thiogalactopyranoside (IPTG), is added (100 mg/l) and the culture is incubated at 37°C for an additional four hours. The cells are centrifuged to pellet them, and the supernatant discarded, and the pellet is stored at –20°C.

Lysis of Bacteria.

To improve reproducibility and yield of protein a commercially available cell lysis reagent is used. First the cell pellet is thawed at room temperature, and then B-Per Bacterial Protein Extraction Reagent (Pierce Endogen, Rockford, IL) was added (25 ml/500 ml) with 1 mM phenylmethanesulfonyl fluoride (PMSF; Sigma, St. Louis, MO), a serine protease inhibitor. The cells are resuspended by vortexing until there were no clumps, and then the vials are shaken at room temperature for 10 minutes, and spun at 27,000 g for 10

minutes. The supernatant is the cell lysate containing the soluble proteins and was stored at 4°C.

Affinity Chromatography.

The protein product is purified by affinity chromatography on a lactosyl-Sepharose column. Bacterial lysates are loaded and the column washed with 5 volumes of phosphate-buffered saline (PBS) with 4-mM 2-mercaptoethanol, 2mM EDTA, and 0.5 mM PMSF. The galectin-3 proteins are eluted with 10 volumes of PBS with 150-mM lactose and 0.5 mM PMSF. The amount of purified protein is determined using the BioRad Protein Assay (Hercules, CA) according to the vendor's instructions.

Enterokinase Digestion.

The *N*-terminal fusion tags are cleaved by digestion with porcine enterokinase enzyme (Sigma) at 0.7 U per mg of galectin-3 protein. The digestion is performed in 20 mM TRIS at pH 5.6 at room temperature for 24-hours.

Derivatization of Cys-delta107 galectin-3 with mPEG2-MAL

The delta-107 protein with an additional Cys residue on the *N*-terminus is derivatized with the mPEG2-maleimide (mPEG2-MAL) molecule (Shearwater Corporation) that has a molecular weight of 40,000, and reacts specifically with thiol groups. A slight molar excess of mPEG2-MAL is added to *N*-terminally truncated galectin-3 (delta107 with an additional single cysteine at the *N*-terminus) with a 10-fold molar excess of lactose at pH 7.5 in PBS at room temperature for two hours. After dialysis to remove the lactose, the PEGylated *N*-terminally truncated protein is purified on affinity chromatography using lactosyl-Sepharose.

Example 3

Polymeric microspheres have been used in formulations of proteins to achieve sustained delivery, and have been approved as part of several different products for humans. Biodegradable poly(lactic-co-glycolic acid) (PLGA) has been widely used as a material for microencapsulation to attain sustained release (88-91). The microspheres are produced by a number of techniques, including freeze-drying or atomizing with gas anti-solvent CO_2 precipitation (92). The resultant nanoparticles are analyzed by scanning electron microscopy.

Encapsulation of delta107 galectin-3 with poly(lactic-co-glycolic) acid microspheres.

A modification of the procedure of Lam *et al.* was used to achieve a product that produced controlled release of nerve growth factor over a period of 14 days (89). Purified delta-107 galectin-3 is formulated at 5 mg/ml in two buffer systems and then lyophilized. The buffer systems can be a 5 mM histidine at pH 5.5 or a 4 mM sodium bicarbonate at pH 7.4 with various concentrations of zinc acetate. Lactose at 5 mg/ml is also added to the buffers. Next, PLGA (1.7 g; 50:50 lactide/glycolide, 12 kDa, RG502H; Boehringer Ingelheim Chemicals (Winchester, VA) is added.

The stability of the lyophilized protein is determined by adding 5 mg of protein from each of the protein formulations to 0.5 ml of ethyl acetate. The samples are homogenized at 8,000 rpm for one minute and then the protein is recovered by dilution into 50-fold excess of 5 mM histidine, pH 5.5. Ultraviolet spectroscopy (280 nm) and affinity chromatography are used to assess the amount and the stability of the protein. The lyophilized formulations containing the greatest amount of recoverable protein are chosen for freeze-drying for encapsulation by PLGA microspheres.

PLGA (1.7 g) is stirred into 10 ml of ethyl acetate at 5°C and then solid delta107-galectin-3 (10% w/w), and zinc carbonate (0-6% w/w), a release modifier, are added. The suspension is homogenized at 8,000 rpm for one minute, and then poured into a 10-ml glass syringe and infused into an

ultrasonic spray nozzle using a pump. The frequency of the spray is 120 kHz into a 2-L round-bottomed flask immersed in a liquid nitrogen bath and containing 300 ml of ethanol frozen underneath liquid nitrogen. The frozen microspheres form upon spraying and then settle on the top of the solid ethanol. The flask is stored at -70°C where the ethanol melted and the ethyl acetate is extracted by the ethanol. After 24 hours, 300 ml of cold ethanol is added, and then after another two days a 20-micron filter is used to removed the ethanol from the microsphere suspension for drying. The microspheres are dried using nitrogen gas at 5°C, and then sieved with a 125-micron stainless steel mesh.

In vivo release is determined by resuspending the microspheres in PBS and incubating them at 37°C in the reservoir of a centrifugal filtration device. After release, the delta107 galectin-3 is obtained by centrifugation, and additional PBS release buffer is added. The integrity of the protein is determined by confirmation of the carbohydrate binding capacity to lactosyl-Sepharose.

Example 4

The carbohydrate-binding sites that are recognized by galectin-3 are widely expressed in tissues and on cells in various species (10, 11, 63, 64). Antibodies that have high affinity for the complex carbohydrates recognized by galectin-3 specifically block the binding of galectin-3 to these sites in the body. Thus, such antibodies have the ability to block metastasis by blocking the multimerization and protein-protein interactions of galectin-3. The effects of the antibodies are similar to the effects obtained with *N*-terminally truncated galectin-3C. In other words, the antibodies reduced metastasis and tumorigencity. However, antibodies that have affinity and specificity to molecules that are commonly expressed in the body are more difficult to produce. A successful alternate approach to use of the carbohydrate ligands as antigens themselves to induce the formation of specific, high-affinity

antibodies is to use phage-display libraries of random peptide sequences to select for peptide mimics of the carbohydrate structures that are then conjugated to highly immunogenic proteins (93-96). Then the peptide conjugates are used to generate antibodies.

Initially, the peptides are expressed as a library in the coat proteins of bacteriophage. Thus, binding to the virion can be used to assess affinity for the peptide expressed. The DNA encoding the peptide of interest can be sequenced readily and, thus, the structure of the peptide determined. Clones of interest are chosen by a process of enrichment called bio-panning. The protein is immobilized on a plate or on beads and incubated with the phage-display library. Unbound phage are washed away, and the bound phage are eluted. The eluted phage are amplified by being taken through the cycle of panning several times to enrich for sequences that bind. Individual clones are characterized by DNA sequencing. Using this approach Ishikawa and Taki (95) derived the sequence of peptides from phage clones that mimicked nLc_4Cer, a ligand of galectin-3 that has a nonreducing terminal *N*-acetyllactosamine unit linked beta1-3 to lactose (paraglobosyl; lacto-*N*-neotetraose).

Bio-panning peptide phage display library for peptide mimetics of carbohydrate ligands of galectin-3.

A library of random peptides (7 or 12-mers) is displayed when fused to a coat protein of M13 Phage. The libraries are available from New England BioLabs (Beverly, MA) and are screened for binding to N-terminally truncated galectin-3 following the instructions of the vendor. The library of 7-mers (Ph.D.- 7) contains 2.0×10^9 independent clones that represent most of the 20^7 of 1.28×10^9 possibilities, and the library of 12-mers (Ph.D. - 12) contains a similar number of independent clones, 1.9×10^9, but this is not exemplary of the possible number of 12-mer sequences (20^{12} or 4.1×10^{15}). The protein can be immobilized to a carboxymethylated dextran-coated biosensor cuvette (Affinity Sensors, Cambridge, U.K.). The phage (2×10^{11}) is reacted with the

immobilized protein for 60 minutes at 25°C in PBS with 0.5% (v/v) Tween-20, and then washed to remove unbound phage. The bound phage is disassociated from the protein by treatment with acidic TRIS-glycine buffer and then neutralized with 1 M TRIS. Phage is amplified by transfection of *E. coli* strain E 3257 that is supplied by New England Biolabs and titered. The phage from this bio-panning is titered and bio-panned a third and a fourth time. Then the phage is plated without amplification on a lawn of *E. coli*, and individual phage (25-30) are picked from the lawn. The DNA is sequenced and the translated peptides for each clone are then determined. Typically, redundant and consensus peptides are derived. The peptides expressed by the greatest number of clones are synthesized.

Detection of binding of galectin-3 to phage by ELISA.

Microtiter wells are coated overnight at 4°C with about 10^{11} CsCl-purified virions, 1 mg of immune-radio assay (I.R.A.) grade bovine serum albumin, or buffer alone. After washing with TRIS-buffered saline with Tween-20, various concentrations of galectin-3 protein is added and the plates incubated for 12 hours at 4°C. After washing three times, monoclonal antibody to galectin-3 (97) conjugated to FITC is added, and the plate incubated for another 1 hour at 37°C and washed again. An ELISA plate reader equipped with fluorescence detection is used to determine the relative degree of binding to the virions. The ability of known carbohydrate ligands of galectin-3 to inhibit the binding can be determined.

Production of humanized monoclonal antibody to galectin-3-binding peptides.

The peptides or consensus peptides with the greatest affinity for galectin-3 can be conjugated to diphtheria toxoid or keyhole limpet hemocyanin and the conjugate can be used to immunize mice or other species. Monoclonal antibodies are produced using methodologies that are standard to those who are skilled in the art (reviewed in (98)). Isolation of B-

cell clones expressing antibody molecules of a desired specificity and affinity are modulated by the immunogenicity of the antigen used for immunization. Size, complexity, and foreignness to the host all contribute to the immunogenicity of a molecule. In general, immunogens must have a molecular mass greater than 10 kDa. Globular proteins tend to be more immunogenic than carbohydrates, lipids, or nucleic acids.

Most peptides are not immunogenic due to their small size so they must be conjugated to carrier proteins such as bovine serum albumin or keyhole limpet hemocyanin. A variety of cross-linking reagents are commercially available for this purpose. The use of peptide conjugates as immunogens results in the expansion of peptide-specific B-cell clones and clones specific for the conjugated or fused protein.

Test bleeds are analyzed before trying to generate the desired monoclonal antibodies because a high titer, specific response of the appropriate immunoglobulin isotype indicates clonal expansion. The immunogenicity of antigen can be enhanced by conjugation to immunogenic carrier molecules, emulsification in adjuvant, or both. Soluble proteins are emulsified in Freunds adjuvant or RIBI Adjuvant System. These adjuvants slowly release antigen over time and induce inflammatory responses. Conjugating soluble antigens to agarose beads or other particulates can stimulate phagocytosis and presentation of antigenic peptides to T-lymphocytes.

The sera from the immunized animal is used to isolate antibody-forming B cells that are fused with a mutant myeloma cell line that has been mutagenized and selected to be defective in hypoxanthine-guanine phosphoribosyltransferase (HGPRT) or thymidine kinase (TK) so that it does not grow in HAT (cell medium containing hypoxanthine, aminopterin, and thymidine). The myeloma cells are deficient in an enzyme required for the salvage pathway of nucleotide synthesis and die in HAT-containing medium. The aminopterin blocks normal nucleotide synthesis and the enzyme deficiency blocks utilization of hypoxanthine or thymidine in the salvage

pathway. When the normal antibody-producing cells from the sera fuse to the mutagenized myeloma cells, the hybrids are able to synthesize DNA and grow in HAT medium. The fused cells are monoclonal antibody producing hybridomas.

The fused hybridomas are selected *in vitro* by growth in HAT medium and then "cloned" so that each well of a microtiter plate contains the progeny of one cell. The supernatants of the cells are screened for the presence of antibody with the desired specificity for peptide mimic and for the complex carbohydrate ligands of galectin-3. Screening can be done using an enzyme-linked immunosorbent assay (ELISA), whole cell ELISA, immunoprecipitation, or immunoblot. Further purification of a hybridoma from a positive well is performed by cloning in soft agar or limiting dilution. Those clones that produce antibody with the desired affinity are expanded for freezing and generation of stock solutions of monoclonal antibody. Monoclonal antibodies then are generated by tissue culture or ascites production, usually in mice. Antibody concentrations in tissue culture supernatants are usually in the range of 10-20 µg/ml, but can be as high as 100 µg/ml. Larger amounts of monoclonal antibody can be generated as ascites fluid in a peritoneal cavity of an animal that is MHC compatible with the myeloma fusion partner and spleen cell donor. As much as 10 mg/ml of monoclonal antibody can be secreted into abdominal ascitic fluid (98).

To produce a therapeutic agent for humans based on a rodent monoclonal antibody, the structure must be humanized to reduce its immunogenicity and prevent the development of human anti-mouse antibodies (99-103). Dozens of rodent antibodies have been humanized, there are many humanized monoclonal antibodies in clinical trials, and now a few of these have been approved as drugs. The rodent complementary determining regions (CDRs) of the antibody are grafted into the human IgG framework. Actually producing the engineered monoclonal antibody using the techniques of molecular biology is relatively simple. The technique is well established but the specificity and affinity of the humanized antibody must be

comparable to the original. If intact IgG is used, this is typically produced in mammalian cells. Antibody fragments such as F(ab')2, Fab', Fab, and scFV are useful as therapeutic agents (104). These are often expressed by bacterial cultures that have shorter expression times, higher yields, and lower costs than mammalian culture systems. Full length IgG has a longer half-life than antibody fragments, but PEGylation of antibody fragments can be used to increase their circulating half-life.

Throughout this application, various publications, including United States patents, are referenced by author and year and patents by number. Full citations for the publications are listed below. The disclosures of these publications and patents in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art to which this invention pertains.

The invention has been described in an illustrative manner, and it is to be understood that the terminology that has been used is intended to be in the nature of words of description rather than of limitation.

Obviously, many modifications and variations of the present invention are possible in light of the above teachings. It is, therefore, to be understood that within the scope of the appended claims, the invention can be practiced otherwise than as specifically described.

LOCUS HUMLAMBP 818 bp mRNA PRI 27-APR-1993
DEFINITION Human non-integrin laminin-binding protein mRNA, complete cds.
ACCESSION M36682
VERSION M36682.1 GI:186921
SOURCE Human breast carcinoma ZR-75-1, cDNA to mRNA, (library of Clontech HL1059a), clones 29K34-43.
ORGANISM Homo sapiens
Eukaryota; Metazoa; Chordata; Craniata; Vertebrata; Euteleostomi; Mammalia; Eutheria; Primates; Catarrhini; Hominidae; Homo.
REFERENCE 1 (bases 1 to 818)
AUTHORS Oda,Y., Leffler,H., Sakakura,Y., Kasai,K.-i. and Barondes,S.H.
TITLE Human breast carcinoma cDNA encoding a galactoside-binding lectin homologous to mouse Mac-2 antigen, Gene 99, 279-283 (1991)
Author address: Y.Oda,
University of California San Francisco, LPPI
Box Bio-0984,
401 Parnassus Avenue,
San Francisco, CA 94143.
FEATURES Location/Qualifiers source 1..818
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VAFHFNPRFNENNRRVIVCNTKLDNNWGREERQSVFPFESGKPFKIQVLVEPDHFK
VAVNDAHL LQYNHRVKKLNEISKLGISGDIDLTSASYTMI"
BASE COUNT 215 a 222 c 212 g 169 t
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61 gcgttatctg ggtctggaaa cccaaaccct caaggatggc ctggcgcatg ggggaaccag
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781 atataatctg aaaggggcag attaaaaaaa aaaacgga

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CLAIMS

What is claimed is:

1. A composition comprising an effective amount of *N*-terminally truncated galectin-3 and a pharmaceutically acceptable carrier.

2. The composition according to claim 1, wherein said *N*-terminally truncated galectin-3 has a sequence according to SEQ ID NO: 1 and analogues thereof.

3. The composition according to claim 1, wherein said *N*-terminally truncated galectin-3 is present in an amount sufficient to reduce tumor size.

4. The composition according to claim 1, wherein said compound is useful for treating cancer.

5. A method of treating a tumor in a patient by administering to a patient in need of treatment an effective amount of *N*-terminally truncated galectin-3 in a pharmaceutically acceptable carrier.

6. The method according to claim 5, wherein said administering step includes intramuscularly administering the *N*-terminally truncated galectin-3.

7. The method according to claim 5, wherein said administering step includes orally administering the *N*-terminally truncated galectin-3.

8. The method according to claim 5, wherein said administering step includes intravenously administering the *N*-terminally truncated galectin-3.

9. The method according to claim 5, wherein said administering step includes locally administering the *N*-terminally truncated galectin-3.

10. An anti-cancer treatment comprising an effective amount of *N*-terminally truncated galectin-3 and a pharmaceutically acceptable carrier.

11. The treatment according to claim 10, wherein said *N*-terminally truncated galectin-3 has a sequence according to SEQ ID NO: 1 and analogues thereof.

12. The treatment according to claim 10, wherein said *N*-terminally truncated galectin-3 is present in an amount sufficient to prevent metastasis.

13. An anti-cancer treatment comprising an effective amount of a nucleic acid sequence encoding an *N*-terminally truncated galectin-3 and a pharmaceutically acceptable carrier.

14. The treatment according to claim 13, wherein said nucleic acid sequence encoding the *N*-terminally truncated galectin-3 is present in an amount sufficient to prevent metastasis.

15. A nucleic acid sequence encoding for an *N*-terminally truncated galectin-3.

16. The nucleic acid sequence according to claim 15, wherein said *N*-terminally truncated galectin-3 has a sequence according to SEQ ID NO: 1 and analogues thereof.

17. A method of treating a tumor in a patient by administering to a patient in need of treatment an effective amount of a nucleic acid sequence encoding an *N*-terminally truncated galectin-3 in a pharmaceutically acceptable carrier.

18. The method according to claim 5, wherein said administering step includes administering the *N*-terminally truncated galectin-3 in a method selected from the group consisting essentially of intramuscularly, orally, intravenously, and locally.

19. An antibody that specifically binds to carbohydrate ligands of galectin-3.

20. The antibody according to claim 19, wherein said antibody is used for treating cancer.

21. An *N*-terminally truncated galectin-3 derivatized with at least one molecule of polyethylene glycol.

22. The derivatized *N*-terminally truncated galectin-3 according to claim 21, wherein said *N*-terminally truncated galectin-3 has added thereto at least one cysteine residue.

FIG. 1

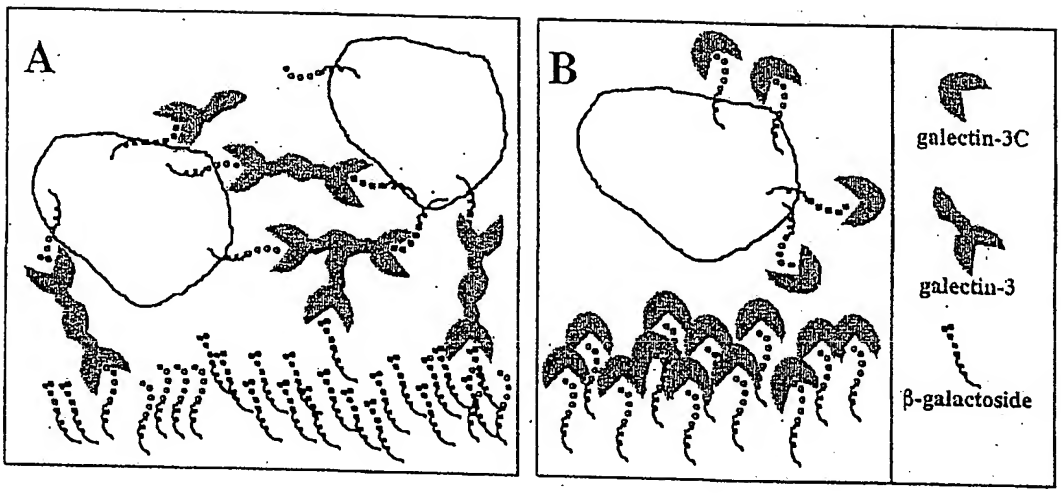


FIG. 2

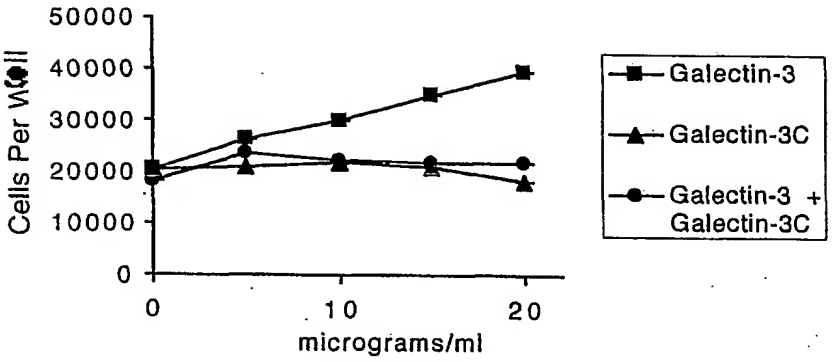

50000
40000
30000
20000
10000
0
Cells Per Well
0
10
20
micrograms/ml
Galectin-3
Galectin-3C
Galectin-3 + Galectin-3C

FIG. 3

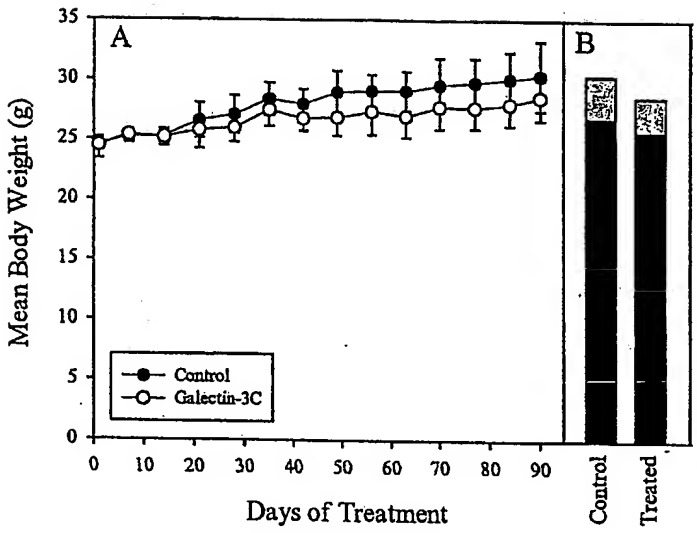

A
B
Mean Body Weight (g)
35
30
25
20
15
10
5
0
0
10
20
30
40
50
60
70
80
90
Days of Treatment
Control
Galectin-3C
Control
Treated

FIG. 4

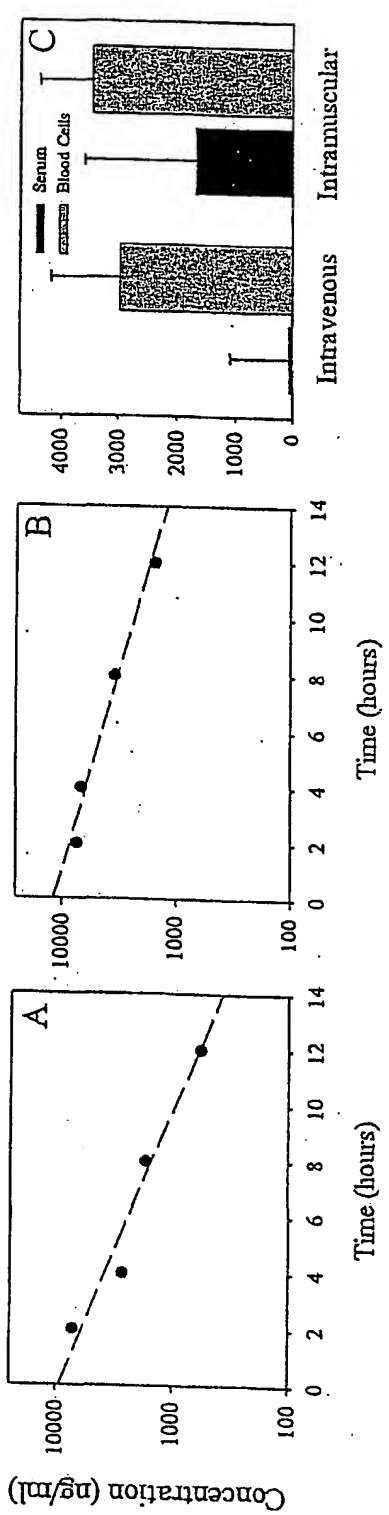

A
B
C
Concentration (ng/ml)
Time (hours)
Time (hours)
Serum
Blood Cells
Intravenous
Intramuscular

FIG.5

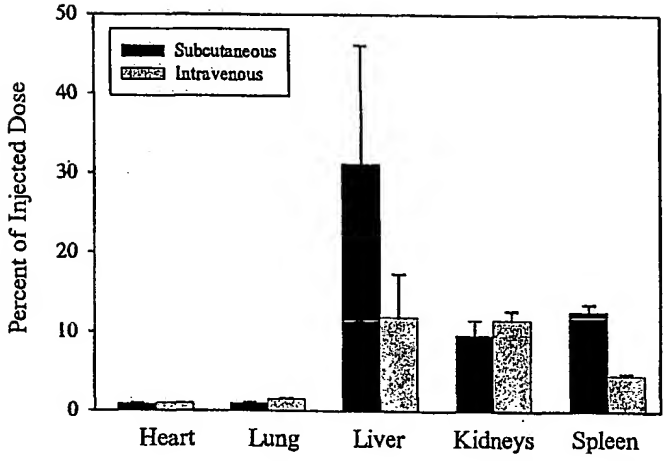

Percent of Injected Dose
50
40
30
20
10
0
Subcutaneous
Intravenous
Heart
Lung
Liver
Kidneys
Spleen

FIG. 6

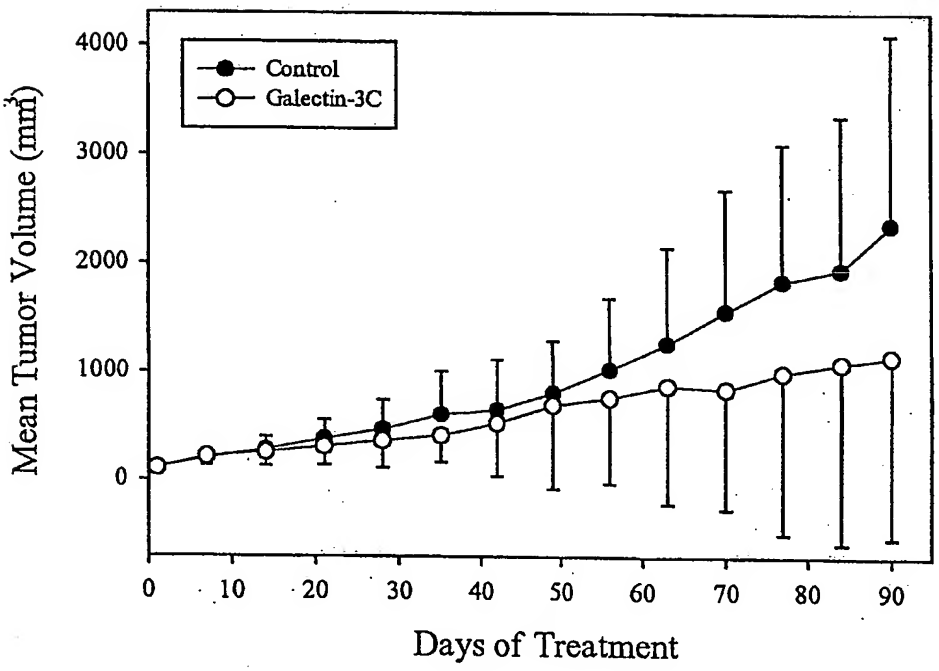

Control
Galectin-3C
Mean Tumor Volume (mm3)
4000
3000
2000
1000
0
0
10
20
30
40
50
60
70
80
90
Days of Treatment

FIG. 7

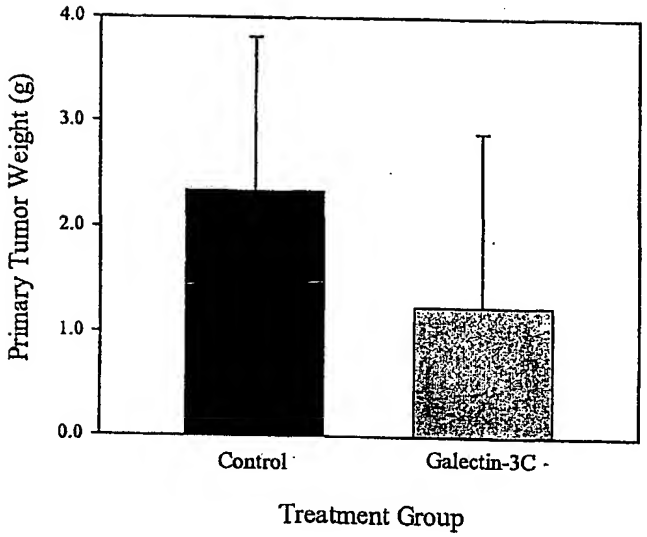

4.0
3.0
2.0
1.0
0.0
Primary Tumor Weight (g)
Control
Galectin-3C
Treatment Group

FIG. 8

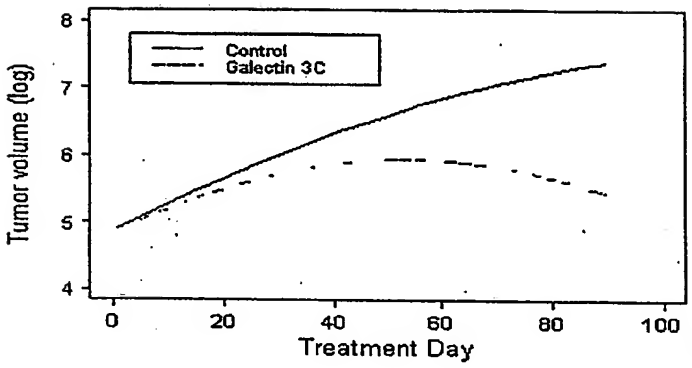

Control
Galectin 3C
Tumor volume (log)
8
7
6
5
4
0
20
40
60
80
100
Treatment Day

FIG. 9

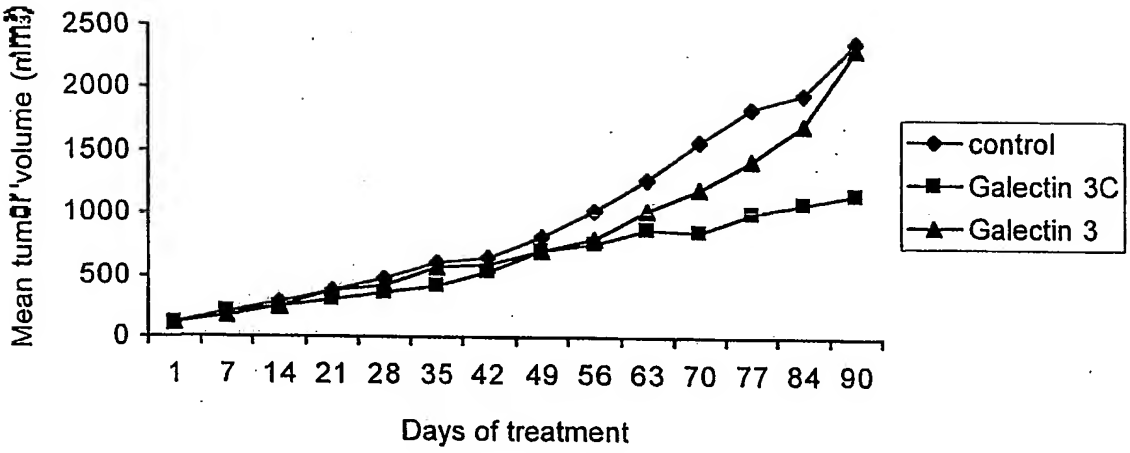
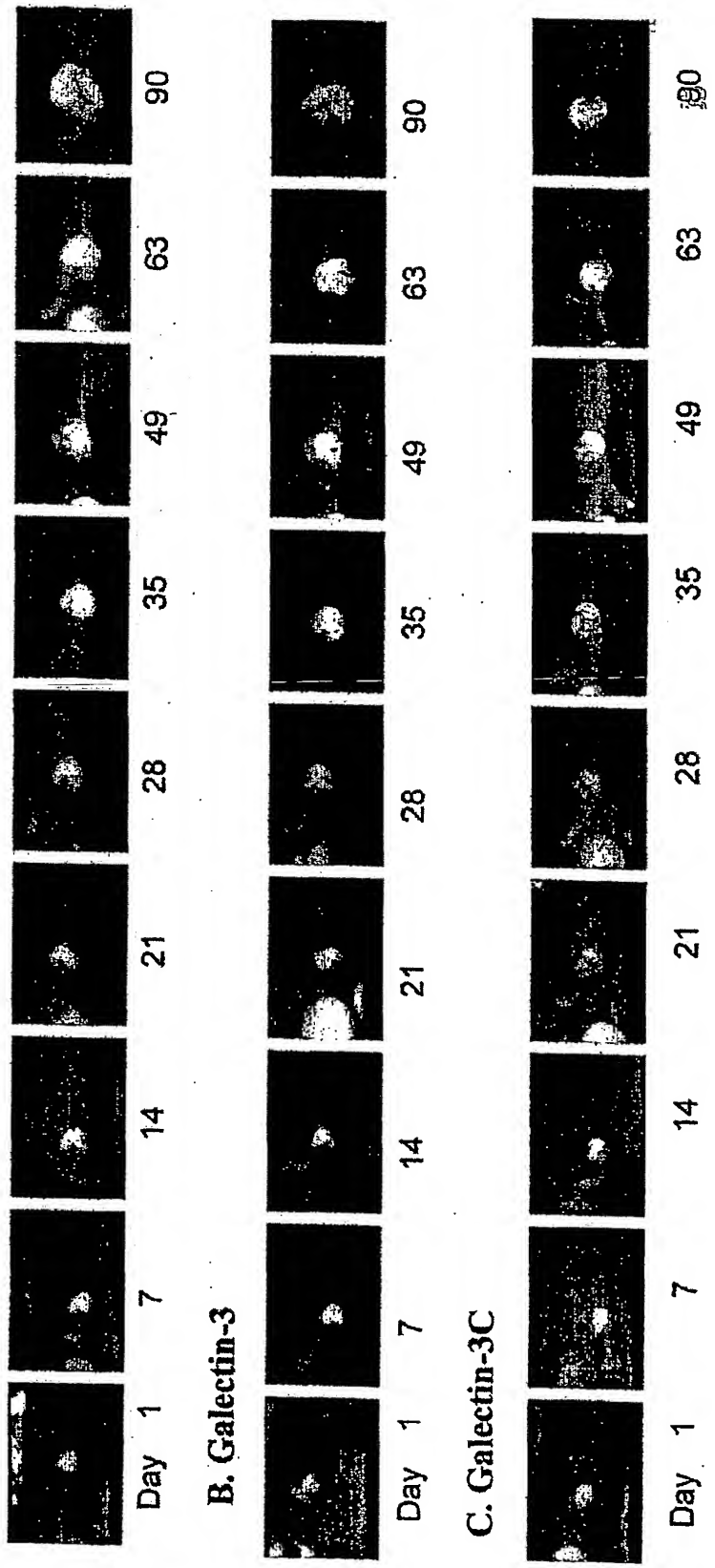

Mean tumor volume (mm3)
2500
2000
1500
1000
500
0
1
7
14
21
28
35
42
49
56
63
70
77
84
90
Days of treatment
control
Galectin 3C
Galectin 3

FIG. 10

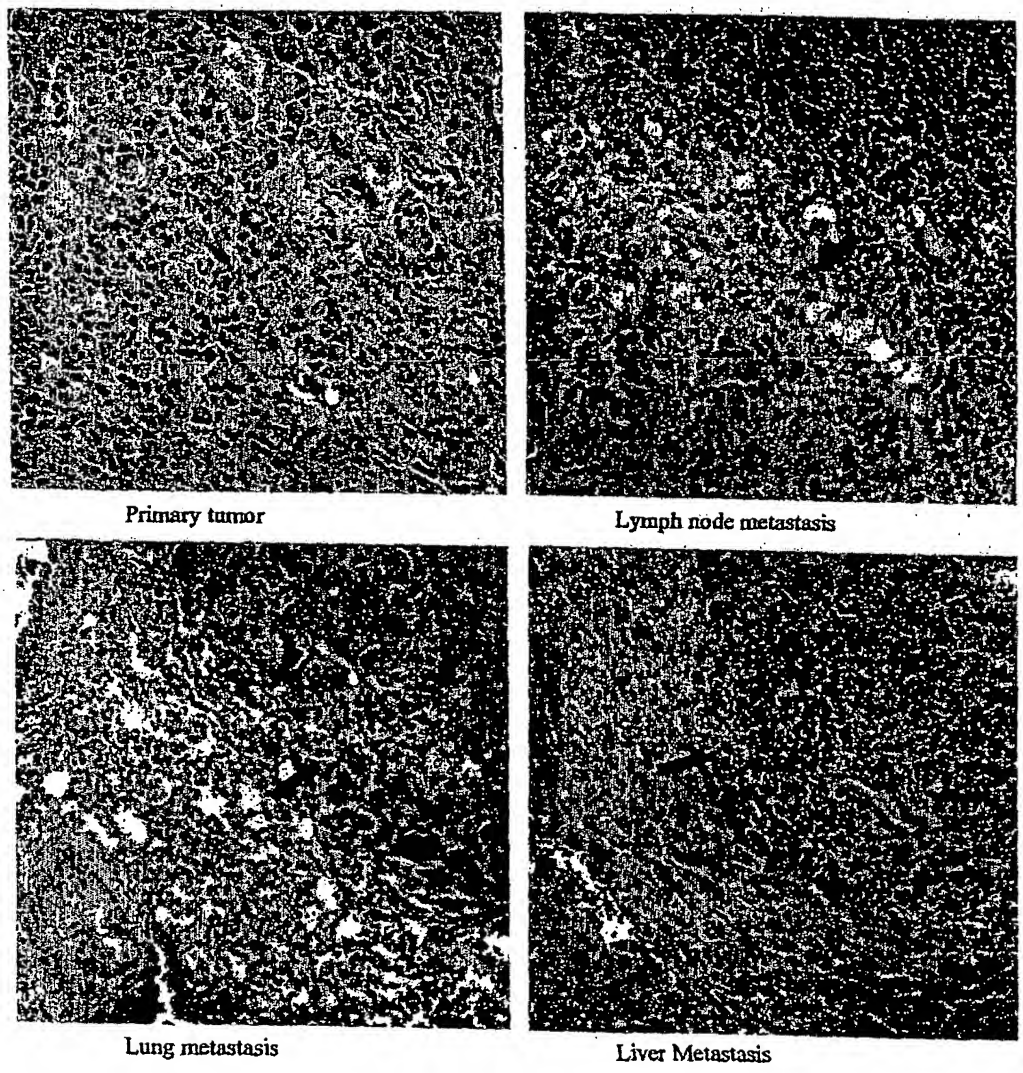


*Typical representative has been chosen for each group

FIG. 11

UCSF-AntiCancer Inc
Efficacy Evaluation of Galectin 3C Against the GFP-Gene Transfected Human Breast Cancer MDA-MB435 in the MetaMouse® Orthotopic Model

Representative of histopathology photos in the control group (sheet 1 of 2)



Primary tumor

Lymph node metastasis

Lung metastasis

Liver Metastasis

FIG. 12

UCSF-AntiCancer Inc
Efficacy Evaluation of Galectin 3C Against the GFP-Gene Transfected Human Breast Cancer MDA-MB435 in the MetaMouse® Orthotopic Model

Representative of histopathology photos in the control group (sheet 2 of 2)

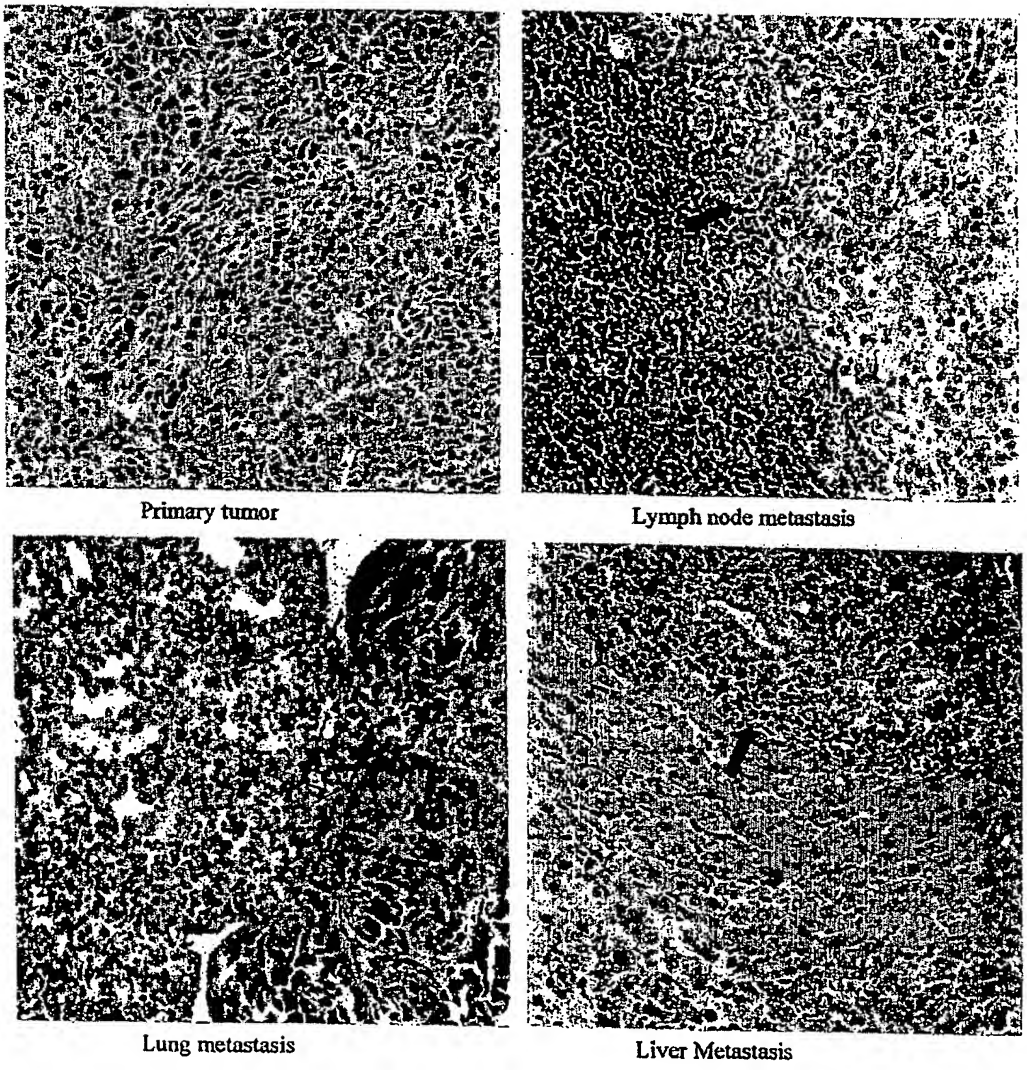
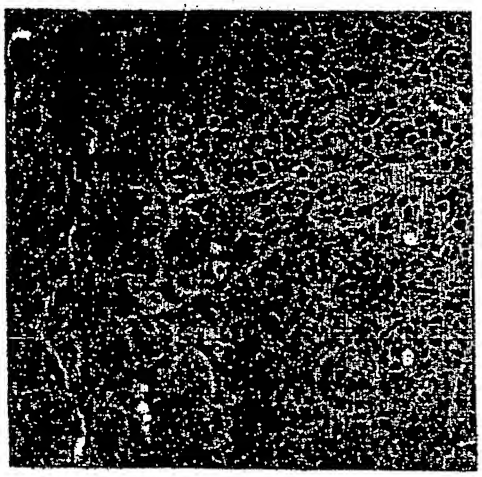


FIG. 13

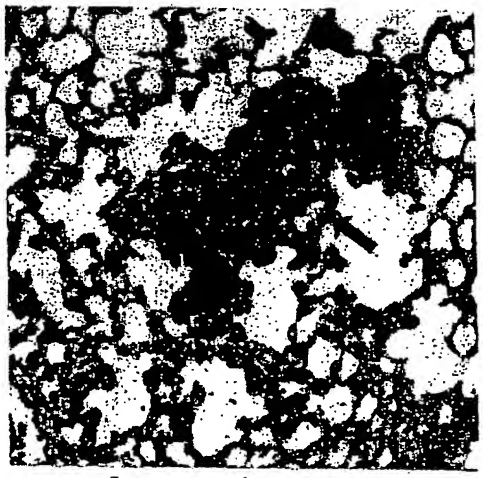
UCSF-AntiCancer Inc
Efficacy Evaluation of Galectin 3C Against the GFP-Gene Transfected Human Breast Cancer MDA-MB435 in the MetaMouse® Orthotopic Model

Representative of histopathology photos in the Galectin 3C group (sheet 1 of 2)



Primary tumor

Lymph node metastasis

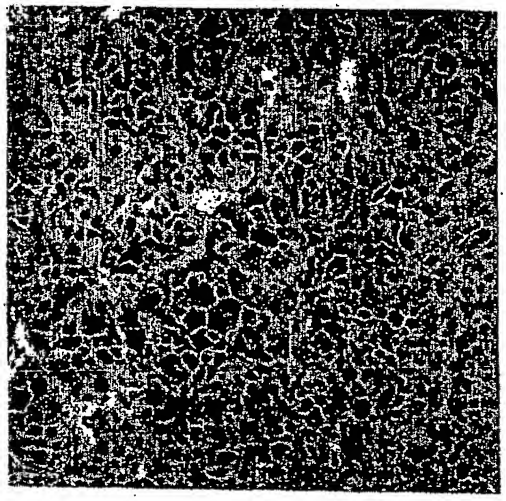


Lung metastasis

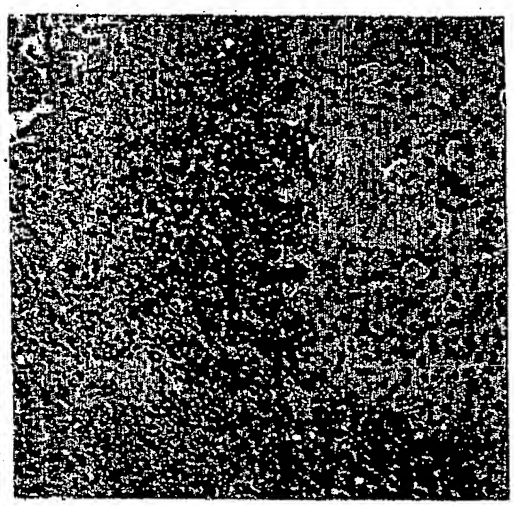
FIG. 14

UCSF-AntiCancer Inc
Efficacy Evaluation of Galectin 3C Against the GFP-Gene Transfected Human Breast Cancer MDA-MB435 in the MetaMouse® Orthotopic Model

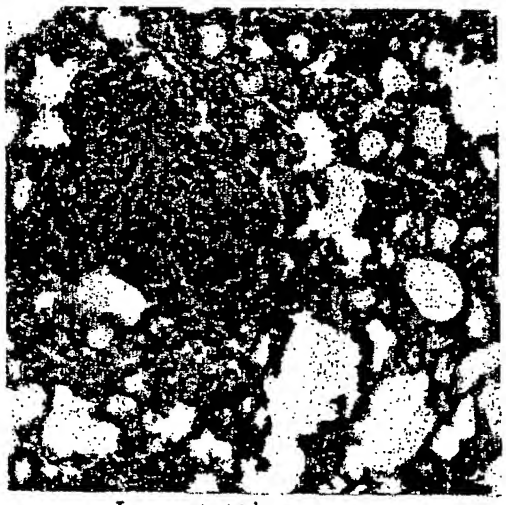
Representative of histopathology photos in the Galectin 3C group (sheet 2 of 2)



Primary tumor



Lymph node metastasis

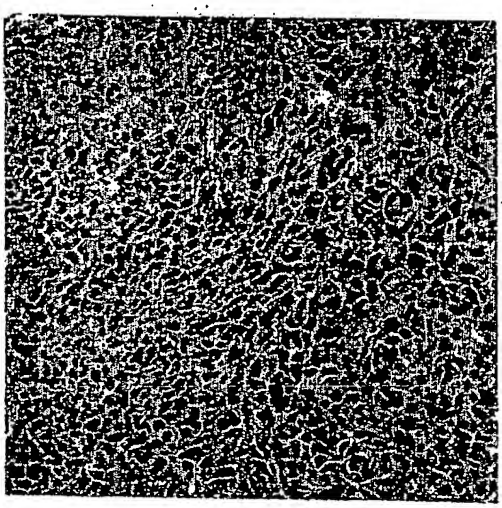


Lung metastasis

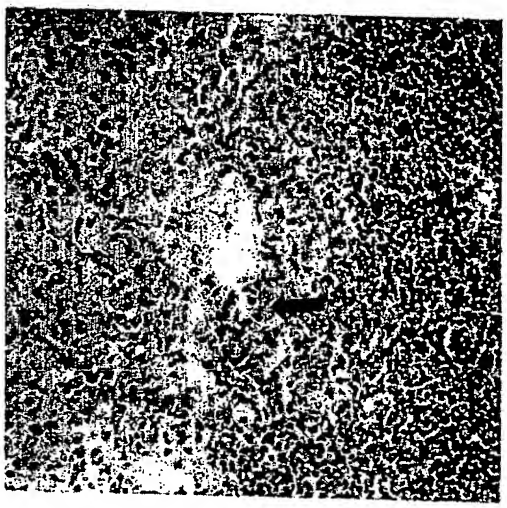
FIG.15

UCSF-AntiCancer Inc
Efficacy Evaluation of Galectin 3C Against the GFP-Gene Transfected Human Breast Cancer MDA-MB435 in the MetaMouse® Orthotopic Model

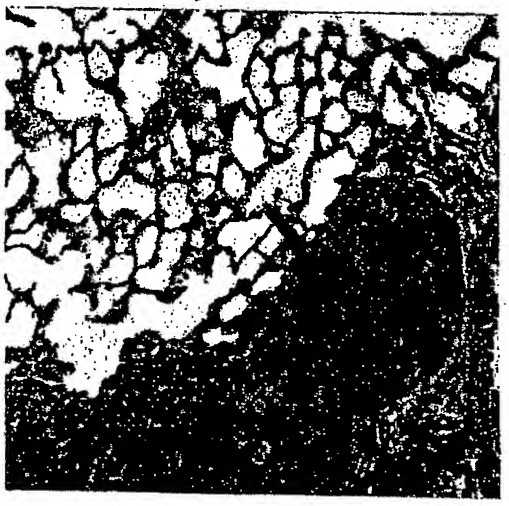
Representative of histopathology photos in the Galectin 3 group (sheet 1 of 1)



Primary tumor



Lymph node metastasis



Lung metastasis

FIG. 16

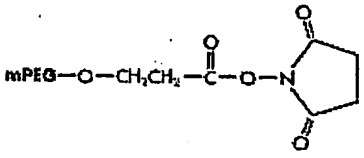
A. Polyethylene glycol

$HO\text{-}(CH_2CH_2O)_nCH_2CH_2\text{-}OH$

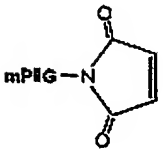
B. Monomethyoxypolyethylene glycol or mPEG

$CH_3O\text{-}(CH_2CH_2O)_nCH_2CH_2\text{-}OH$

C. mPEG-Succinimidyl Propionate (mPEG-SPA)



D. mPEG-Maleimide (mPEG-MAL)



E. mPEG2-Maleimide (mPEG2-MAL)

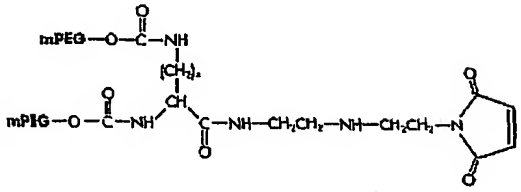


FIG. 17